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Molecular and biotechnological approaches to essential oils production in *Thymus caespititius*

Marta Daniela de Sá Mendes

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“The important thing in science
is not so much to obtain new facts
as to discover new ways of thinking about them”

Sir William Henry Bragg

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"Friendship is unnecessary, like philosophy, like art…
It has no survival value;
rather it is one of those things which give value to survival"
C.S. Lewis

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Obrigadíssima

List of nonstandard abbreviations

aa – amino acid

BA – 6-Benzyladenine

CTAB - Cetyl trimethyl ammonium bromide

DMAPP - dimethylallyl diphosphate

FE – Fungal extracts

FPP - farnesyl diphosphate

fw – fresh weight

IBA - Indole-3-butyric acid

GA - Glutaraldehyde

GC - Gas Chromatography

GC-MS - Gas Chromatography-Mass Spectrometry

GGPP - geranylgeranyl diphosphate

GPP - geranyl diphosphate

IPP - isopentenyl diphosphate

LB - Luria–Bertani

MAV - mevalonic acid pathway

MEP - methylerythritol phosphate pathway

MS – Murashige and Skoog

ORF – Open reading frame

PDA – Potato dextrose agar

3'RACE-PCR – 3' Rapid amplification of cDNA ends PCR

SEM - Scanning Electron Microscopy

SH - Schenk and Hildebrandt

SPME – Solid phase micro extraction

Tctps – *Thymus caespititius* terpene synthase

Tctps2 – *Thymus caespititius* terpene synthase 2

Tctps4 – *Thymus caespititius* terpene synthase 4

Tctps5 – *Thymus caespititius* terpene synthase 45

TPS – Terpene synthase

3'-UTR – 3'-Untranslated region

5'-UTR – 5'-Untranslated region

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Summary

Thymus caespititius is an aromatic plant from the NW Iberian Peninsula and from the Azores and Madeira archipelagos. In this species chemical polymorphism in the essential oils has been described, with seven well defined chemotypes, namely thymol, carvacrol, α-terpineol, sabinene, and the mixed chemotypes thymol/sabinene, thymol/carvacrol and thymol/sabinene/carvacrol. *T. caespititius* essential oils already proved to have antimicrobial, antioxidant activity and acetylcholinesterase inhibition, and more recently they were shown to have high nematicidal activity against the pinewood nematode *Bursaphelenchus xylophilus*, namely the carvacrol and thymol rich oils. Since this species is not used commercially, the oils diversity observed results only from natural evolution; however, the molecular mechanisms underlying such chemical diversity are not yet clear.

In this work were used molecular and biotechnological approaches to understand the basis for chemical diversity of *T. caespititius'* essential oils.

Five *T. caespititius in vitro* cultures from plants with different chemotypes were established, allowing having enough plant material for the following studies. This advantageous *in vitro* system avoids plant material collection from the natural habitat and the controlled growth conditions reduce the sources of variability known to affect essential oil composition.

Terpenes are the main group components identified in *T. caespititius* essential oils. However the molecular basis of their synthesis has not yet been fully understood. Given this, the genes encoding enzymes of monoterpene biosynthesis were searched and identified in the five established *in vitro* genotypes and latter their expression level was assessed.

It has been shown that biotic (pathogens, herbivores) and abiotic (temperature, light quality, nutrients type and availability) factors could influence the essential oil composition as well as the genes involved in their biosynthesis. So, nutrient composition and *Botrytis cinerea* fungal extracts were assayed in *in vitro* cultures to study the biosynthesis and accumulation of terpenes in this aromatic species.

Collectively, it was demonstrated that the conditions established in this work also step up the possibility of using *T. caespititius* shoot cultures as an experimental model to investigate the terpene biosynthesis and accumulation in plants. The large biomass increase obtained *in vitro* advance the possibility of using large-scale *in vitro* production of desirable secondary metabolites. The terpene synthase genes identified and characterized here were a small contribution to understanding how these genes evolved, are regulated and function in Lamiaceae

Keywords: Lamiaceae, *Thymus caespititius*, thyme, chemotype, *in vitro* cultures, monoterpene, terpene synthase, elicitation, nutrient composition, *Botrytis cinerea.*

Resumo

Thymus caespititius é uma planta aromática nativa do NO da Península Ibérica e dos arquipélagos dos Açores e da Madeira. Nos óleos essenciais desta espécie foi encontrado polimorfismo químico, tendo sido definidos sete quimiotipos, nomeadamente timol, carvacrol, α-terpineol, sabineno, timol/sabineno, timol/carvacrol e timol/sabineno/carvacrol. Em estudos anteriores, os óleos essenciais de *T. caespititius* revelaram ter actividade antimicrobiana, antioxidante, anticolinesterásica e mais recentemente mostraram elevada actividade nematicida contra o nemátode da madeira do pinheiro (*Bursaphelenchus xylophilus*), principalmente os óleos ricos em carvacrol e timol. Uma vez que esta espécie de tomilhos não está disponível comercialmente, a diversidade observada nos óleos visível mesmo em plantas cultivadas nas mesmas condições ambientais são resultado de evolução natural e não de selecção. Contudo os mecanismos moleculares envolvidos nesta diversidade química não são ainda claros.

Neste trabalho, foram utilizadas abordagens moleculares e biotecnológicas de modo a compreender e esclarecer a diversidade química dos óleos essenciais de *T. caespititius.*

A utilização de culturas *in vitro* em estudos de produção de óleos essenciais tem sido explorada em várias espécies aromáticas. A utilização deste sistema é vantajoso, pois não só evita a recolha de material vegetal do seu habitat natural, como também permite, pelas condições de crescimento controladas, reduzir as fontes de variabilidade que afectam a composição dos óleos essenciais.

No presente trabalho de investigação, cinco genótipos foram estabelecidos *in vitro*, três com os quimiotipos carvacrol (C), carvacrol/timol (CT) e sabineno/carvacrol (SC) e dois resultantes de uma planta mãe com o quimiotipo α-terpineol (G1 e G2). Os óleos essenciais destes genótipos foram avaliados em culturas de rebentos em proliferação (6 a 24 repicagens após o estabelecimento *in vitro*) e comparados com os das respectivas plantas de campo. Os óleos essenciais foram isolados por hidrodestilação e analisados por GC e GC-MS. As culturas *in vitro* mantiveram estável a composição dos óleos essenciais durante os dois anos de análise. As culturas resultantes de gemas axilares (C, CT e SC) produziram óleo qualitativamente semelhante ao das plantas correspondentes em campo, apesar de pequenas diferenças quantitativas. O genótipo C revelou um perfil químico semelhante ao da planta de campo, sendo o carvacrol (20-69% nos rebentos e 71% na planta de campo) o componente maioritário dos óleos, seguido pelo acetato de carvacrilo (6-40% nas culturas *in vitro* e 4% na planta de campo). Para o genótipo CT, os componentes maioritários detectados na planta de campo foram o carvacrol (42%) e o timol (23%); enquanto nas culturas *in vitro* os componentes maioritários detectados foram o carvacrol (16-28%), o timol (17-25%), o acetato de

carvacrilo (11-23%) e o acetato de timilo (9-15%). No genótipo SC, os componentes dominantes dos óleos essenciais tanto das plantas de campo como das culturas *in vitro* foram o carvacrol (11-28%), o sabineno (18-49%), e o timol (8-12%).

As culturas *in vitro* resultantes de sementes (G1 e G2) apresentaram perfis de óleos essenciais diferentes dos da planta mãe (quimiotipo α-terpineol), esta diferença pode dever-se à variabilidade genética das sementes resultantes de cruzamentos abertos. Para G1 e G2 foi impossível determinar o seu quimiotipo. Nestes genótipos foram detectados baixos níveis de α-terpineol e o inverso foi observado para o timol, o carvacrol metil éter, o acetato de timilo e o carvacrol.

O aumento de biomassa obtido com as culturas *in vitro* permitiu estudar a biossíntese dos terpenos e a sua regulação sob factores bióticos e abióticos.

Os óleos essenciais de *T. caespititius* são uma mistura complexa de compostos, na sua grande maioria de natureza terpénica. No entanto, as bases moleculares da biossíntese destes compostos ainda não foram completamente esclarecidas. Posto isto, utilizaram-se as culturas *in vitro* de *T. caespititius* para procurar os genes envolvidos na biossíntese dos terpenos, nomeadamente os genes das terpeno sintase (TPS). Foram identificados e caracterizados três genes *Tctps* (*Tctps2*, *Tctps4* e *Tctps5*) neste sistema; dois dos quais, *Tctps2* e *Tctps5*, haviam já sido identificados em plantas de campo. O gene *Tctps5* foi apenas detectado nos genótipos G1 e G2. Com o alinhamento dos três genes *Tctps* detectou-se que o *Tctps4* não possuía péptido-sinal ou este era muito pequeno (3-4 aa) em comparação com *Tctps2* e *Tctps5* (46-47 aa). A grelha de leitura deste gene (1665 bp) é também menor que a dos genes *Tctps2* (1794 bp) e *Tctps5* (1806 bp). O alinhamento das sequências de aminoácidos das três terpeno sintases revelou que estas partilhavam 77% de semelhança.

Tendo em conta a informação disponível das terpeno sintases de espécies da família Lamiaceae, o tamanho do gene *Tctps4* sugeria que provavelmente este gene codificava uma sesquiterpeno sintase. Contudo, os ensaios enzimáticos das Tctps com o substrato GPP revelaram que tanto *Tctps2* como *Tctps4* codificavam para a γ-terpineno sintase, enquanto *Tctps5* codificava para uma α-terpineol sintase. Estas proteínas não revelaram qualquer actividade sesquiterpénica em ensaios com FPP como substracto. Os ensaios enzimáticos com os extractos proteicos foram realizados a três temperaturas diferentes, 4º, 21º e 42ºC. Apesar destes dados serem apenas qualitativos, a temperatura não pareceu afectar a actividade da Tctps4, enquanto a 4ºC a actividade de Tctps2 isolada do genótipo C foi muito reduzida, sendo retomada com o aumento da temperatura. Nos restantes ensaios com Tctps2 não foram detectadas grandes diferenças de actividade. Quanto à Tctps5 pareceu existir uma variação no produto relacionada com a temperatura. Em baixas temperaturas o produto maioritário formado foi o limoneno, enquanto a 21ºC e 42ºC formou-se

predominantemente o α-terpineol.

A existência de dois isogenes para γ-terpineno sintase, sugere duplicação de genes durante o processo evolutivo, seguido de mutações levando à diferenciação dos dois genes, não comprometendo, no entanto, a actividade da proteína. É de salientar que nas plantas de *T. caespititius* analisadas, o gene *Tctps4* encontrava-se menos expresso que o *Tctps2*. Nas plântulas *in vitro* foi detectada uma elevada acumulação de transcritos dos genes *Tctps*, ao contrário do verificado nas raízes. Este resultado era expectável, uma vez que, a síntese e acumulação dos terpenos em Lamiaceae ocorrem em células especializadas, como os tricomas glandulares existentes na parte aérea. A quantidade de γ-terpineno encontrada nos óleos essenciais de *T. caespititius* foi reduzida, o que poderá dever-se à sua rápida conversão em carvacrol e timol, dois dos componentes maioritários dos óleos desta espécie.

Os estudos de mutagénese dirigida em Tctps2 demonstraram que o resíduo Arg-505 (R505G) é essencial para a estabilidade e/ou correcto enrolamento da proteína e a alteração desse aa por um aa hidrofóbico não-polar (Gly) quebrou essa estabilidade. A inserção de duas outras mutações neste gene (D405G e A521V) não revelou efeito na actividade da proteína.

Ao longo dos anos, tem-se verificado que tanto os óleos essenciais como os genes envolvidos na sua biossíntese são influenciados por factores bióticos (agentes patogénicos, herbívoros) e abióticos (temperatura, qualidade da luz, tipo e disponibilidade de nutrientes). Por este motivo foram feitos ensaios nas culturas *in vitro* de *T. caespititius* para avaliar o efeito de diversos factores na biossíntese e acumulação dos terpenos.

Os cinco genótipos estabelecidos *in vitro* foram colocados a crescer em dois meios de cultura, MS e SH, de forma a testar o efeito da composição de nutrientes. Nas culturas crescidas em SH observou-se maior rendimento de óleo (0.3-3.4%) do que nas culturas crescidas em MS (0.3-1.2%). Os óleos essenciais das plantas crescidas nas duas condições foram qualitativamente semelhantes. Contudo, observaram-se algumas diferenças quantitativas, principalmente nos compostos com o grupo acetato, acetato de timilo e acetato de carvacrilo, cuja quantidade relativa diminuiu nos rebentos crescidos em SH. Os componentes maioritários dos óleos isolados do genótipo C foram o carvacrol (25-60%) e o acetate de carvacrilo (9-45%). As plantas crescidas em SH continham menos acetato de carvacrilo (9-13%) do que as plantas desenvolvidas em MS (23-45%). Quanto aos óleos do genótipo CT, detectou-se elevada percentagem de carvarol (13-28%), timol (14-23%), acetato de carvacrilo (10-26%) e acetato de timilo (8-18%). Nestes óleos também se observou uma diminuição da quantidade relativa nos compostos com o grupo acetato. Nas culturas SC, os componentes maioritários dos óleos foram o sabineno (35-45%), o carvacrol (11-19%) e o timol (8-11%). Já nos

óleos essenciais dos genótipos G1 e G2, os componentes identificados em maior concentração foram o *p*-cimeno (7-34%), o γ-terpineno (4-21%), o timol (4-18%) e o carvacrol metil éter (4-17%). Nas plantas crescidas em SH observou-se uma diminuição na quantidade relativa de carvacrol metil éter (4-8%), sendo o inverso observado no *p*-cimeno (10-34%) e no γ-terpineno (6-21%). Em G2, também se observou diminuição na quantidade de timol (4-7%).

A acumulação dos transcritos das terpeno sintase (*Tctps2* e *Tctps4*) foi superior nas culturas *in vitro* desenvolvidas em SH, excepto no genótipo SC. Em G1 e G2, o gene *Tctps4* apresentou-se menos expresso que nos restantes genótipos. Quanto ao gene *Tctps5* a sua expressão foi apenas detectada em G1 e G2; culturas onde se observou maior quantidade de α-terpineol.

Entre os dois meios de cultura testados observam-se várias diferenças na sua composição nutricional, sendo a concentração de azoto e a razão NH_4^+/NO^{3-} as mais notórias. Estes poderão estar relacionados com as diferenças observadas na composição dos óleos essenciais e na expressão dos genes das terpeno sintase. No entanto, será necessário confirmar esta hipótese, testando diferentes concentrações de azoto e razões NH_4^+/NO^{3-} em MS.

Os extractos fúngicos de *Botrytis cinerea* não afectaram qualitativamente nem quantitativamente os óleos de CT, no entanto em SC, observou-se diminuição do conteúdo de sabineno e aumento dos compostos com actividade antifúngica, carvacrol e timol.

A expressão dos genes *Tctps2* e *Tctps4*, avaliada ao longo de 30 dias não revelou diferenças entre controlo e *stress* (presença de extracto de *B. cinerea*). No genótipo SC, os extractos fúngicos aumentaram cerca de 6x a expressão dos genes *Tctps* nas primeiras horas de tratamento. Enquanto em CT apenas se observou um ligeiro aumento (aproximadamente 2.8x). Nos restantes pontos de análise observou-se diminuição da regulação destes genes (cerca de 1.5-15.7x nas culturas CT e 1.0-3.5x nas culturas SC), o que pode estar relacionado com a translocação dos recursos energéticos para actividades vitais da planta.

Neste trabalho foi possível demonstrar que as culturas *in vitro* de *T. caespititius* são um bom modelo experimental para a investigação da biossíntese e acumulação de terpenos. A identificação e caracterização dos genes das terpeno sintase neste trabalho permitem contribuir para a compreensão da função, regulação e evolução destes na família Lamiaceae.

Keywords: Lamiaceae, *Thymus caespititius*, tomilho, quimiotipo, culturas *in vitro*, monoterpenos, terpeno sintase, eliciação, composição de nutrientes, *Botrytis cinerea.*

Chapter I

General Introduction

1. State of the art

Plants produce a high diversity of secondary metabolites. These compounds play a role in the plant's survival in the ecosystem. The secondary metabolites are involved in diseases and pest resistance, pollinator's attraction, in the relation between microorganisms, among others. The capability to produce the secondary metabolites has been selected trough the evolution processes by the different plant species, according to their requirements. The chemical solutions for a common problem are generally diverse for the different species.

During the last decades the economic interest of secondary metabolites has increased. However, the progress in this research area is still limited, because of the lack of knowledge about the biosynthetic pathways (genetics and biochemistry) of secondary metabolites.

1.1. Lamiaceae family

Lamiaceae (Labiatae), also known as the mint family, is a family of flowering plants. The original family name was Labiatae, so given because the flowers typically have petals fused into an upper lip and a lower lip. Although this is still considered an acceptable alternative name, most botanists now refer to the Lamiaceae. This family of plants comprises approximately 251 genera and 6970 species (Judd et al. 2002; Simpson 2006).

The Lamiaceae are distinctive in being herbs or shrubs, often aromatic with ethereal oils, with usually 4-sided stems, opposite (or whorled) leaves, a verticillaster or thyrse inflorescence (flowers solitary and axillary in some), and zygomorphic (rarely actinomorphic) usually bilabiate flowers having a superior ovary, often deeply 4-lobed (by formation of false septa) with a gynobasic style, the fruit being a schizocarp of usually 4 nutlets or a berry or drupe (Simpson 2006).

The Lamiaceae have a mostly worldwide distribution. Economic importance includes medicinal plants, culinary herbs (e.g., *Mentha*, mint; *Ocimum*, basil; *Rosmarinus*, rosemary; *Salvia*, sage; *Thymus*, thyme), and fragrance plants (e.g., *Lavandula*, lavender; *Pogostemon*, patchouli) (Simpson 2006).

Essential oil biosynthesis, which is produced by glandular trichomes, is one of the characteristic features of the Lamiaceae family. This plant family has nonglandular and glandular trichome types on the aerial parts in the different species, such as in basil, mint, oregano and thyme. The nonglandular trichomes may be conical unicellular and/or multicelular bent point-shaped-type. The glandular trichomes are of two main types: peltate and two types of capitate (subtype I and II). Peltate trichomes consist of a short stalk cell and a large head with a variable even-number of cells (12-14) arranged in

two concentrical rings (the inner ring with four cells and the outer ring with eight to ten cells). The secreted material accumulates in the subcuticular space and it is released after cuticle rupture, following a fragility line in the medium region of the glandular head. Both capitate trichomes subtypes (I and II) are composed of a stalk, a short neck and a unicellular head cell, differing only in the size of the head, subtype I trichomes having a larger ovoid head. The secretion accumulates in a small subcuticular space (Gershenzon et al. 1989; Gershenzon et al. 1992; Werker et al. 1993; McCaskill and Croteau 1995; Bohlmann et al. 1998; Turner et al. 1999; Gang et al. 2001; Figueiredo et al. 2008a; Mendes et al. 2010, Lange and Turner 2013).

1.1.1. *Thymus caespititius*

Thymus caespititius Brot. (Fig. 1) is a low creeping shrub, with caespitose habit and pink purplish or whitish inflorescences, which belongs to the section Micantes of the genus *Thymus* (Morales 2002). Being characteristic of Atlantic wet areas, *T. caespititius* is an endemic plant of the northwest Iberian Peninsula and of the Madeira and Azores archipelagos (Pereira et al. 2000).*T. caespititius* Brot. is commonly known as “erva-úrsula”, “alecrim-da-serra” or “tormentelo” (Schäfer 2005). This species is the only representative of the genus in this archipelago, and it is one of the few Azorean plants with an altitude range extending from sea level to the highest altitudes, as in Pico Island (Pereira et al. 2000).

Figure 1. *Thymus caespititius* growing in small patches in the wild. Bars = 1 cm.

The genus *Thymus* is a taxonomically complex group of aromatic plants used all over the world with many purposes, due to their aromatic and medicinal properties and also as a culinary herb (Salgueiro 2006). Thyme medicinal usage is due to its antiseptic, anti-spasmodic, expectorant, and antioxidant activities, among others (Zarzuelo and Crespo 2002; Salgueiro 2006; Figueiredo et al. 2008a).

Previous studies on essential oils of this species (Table 1) revealed the existence of chemical polymorphism, with seven well-defined chemotypes, carvacrol, thymol, α-terpineol, sabinene, carvacrol/α-terpineol, α-terpineol/τ-cadinol and carvacrol/thymol (Salgueiro 2006; Figueiredo et al. 2008a; Trindade et al. 2008). The α-terpineol chemotype is the only found in mainland Portugal and in Madeira Island.

Table 1. Main components of *T. caespititius* essential oils identified in several studies.

Collection site	Main components (%)	Reference
Mainland Portugal	α-Terpineol (31-41); *p*-Cymene (6-9); τ-Cadinol (6-9)	Salgueiro et al.1997
Pico Island	Carvacrol (36); Thymol (16); Carvacryl acetate (8)	
São Jorge Island	α-Terpineol (t-68); Thymol (1-58); Carvarol (1-52) Sabinene (0-41)	Pereira et al.2000
Pico Island	Carvacrol (45-57); Thymol (2-12); Carvacryl acetate (2-17)	Pereira et al.2003
Faial Island	Carvacrol (51-54); α-Terpineol (5-12)	
Graciosa Island	α-Terpineol (15-38); Carvacrol (3-35); τ-Cadinol (4-11)	
Trás-os-Montes	α-Terpineol (32)	Miguel et al.2004
Corvo Island		Santos et al.2005
Flores Island	Carvacrol (41-65); Carvacryl acetate (2-24); *p*-Cymene (4-19); τ-Cadinol (t-15)	
São Miguel Island		
Terceira Island	Thymol (35-51); Thymyl acetate (10-19) *p*-Cymene (10-14)	
Madeira Island	α-Terpineol (33-37) Sabinene (8-16); β-Myrcene (7-10)	
São Miguel Island	Carvacrol (45-68); Carvacryl acetate (5-23)	Mendes 2007
Faial Island	Carvacrol (47-65); Thymol (1-13)	
Madeira Island	α-Terpineol (33)	
Pico Island	Carvacrol (4-63); Thymol (t-56); Carvacryl acetate (1-17)	Trindade et al.2008
São Jorge Island	Sabinene (t-72); α-Terpineol(t-58); Carvacrol (t-58); Thymol (t-34); τ-Cadinol	
Terceira Island	Thymol (22-54); Thymyl acetate (5-19); *p*-Cymene (8-13)	
Corvo Island	Carvacrol (17-53); Carvacryl acetate (6-22); τ-Cadinol (4-15)	Trindade et al.2009
Flores Island	Carvacrol (37-66); τ-Cadinol (4-15); Carvacryl acetate (3-14); *p*-Cymene (4-12)	
Graciosa Island	α-Terpineol (7-53); Carvacrol (t-35); Thymol (t-23); τ-Cadinol (3-16); *p*-Cymene	
Terceira Island	Thymol (13-46); Carvacrol (3-42); *p*-Cymene (10-12); Thymyl acetate (2-11)	Lima 2009; Lima et al.2010
São Jorge Island	Carvacrol (t-44); Sabinene (t-42); α-Terpineol (t-42); Thymol (t-33); Carvacryl	
Flores Island	Carvacrol (40-63); *p*-Cymene (7-12); τ-Cadinol (3-12)	
Graciosa Island	α-Terpineol (19-37); Carvacrol (8-13); τ-Cadinol (8-12); γ-Terpinene (7-11)	
Mainland Portugal	α-Terpineol (24-42); γ-Terpinene (12-14); *p*-Cymene (8-12)	
Northwest Spain	α-Terpineol (26); *p*-Cymene (25)	Salas et al.2011

t - trace (≤ 0.05%);

T. caespititius essential oils showed high levels of antimicrobial (Dandlen et al. 2011a), antioxidant (Miguel et al. 2003; Miguel et al. 2004; Dandlen et al. 2010), acetylcholinesterase inhibition (Dandlen et al. 2011b) activities. Recently, some of *T. caespititius* essential oils, principally those carvacrol- and thymol-rich, showed high nematicidal activity against the pinewood nematode *Bursaphelenchus xylophilus* (Barbosa et al. 2010; Barbosa et al. 2012; Faria et al. 2013). In forest ecosystems, this nematode is considered one of the most important pathogens of coniferous forests worldwide (Mota

and Vieira 2008), being detected in Portugal in *Pinus pinaster,* and causing both economic damage to the forest and forest products industries.

The chemical variability observed in *T. caespititius* could reflect a genetic diversity possibly related to the heterogeneity of environmental conditions such as humidity degree, thermal amplitude and soil type (Figueiredo et al. 2008b; Lima 2009). Here upon, few studies were made in order to establish a relation between the chemical profile of the essential oils and the molecular diversity of *T. caespititius* detected by random molecular markers (Mendes 2007, Trindade et al. 2008; Lima 2009; Trindade et al. 2009; Lima et al. 2010). In Mendes (2007), a moderate correlation between the chemical and molecular diversity was found and discriminated in different groups the plant individuals from Madeira and Azores archipelagos. In the others studies, no direct correlation was observed between chemotypes and DNA profiles, since the individuals were grouped differently in the clusters (Trindade et al. 2008; Lima 2009; Trindade et al. 2009; Lima et al. 2010).

Random markers did not help to explain the chemical polymorphism observed in this species. So, a more direct approach, involving the study of terpene synthase (TPS) genes, was conducted to elucidate the metabolic pathways involved in the synthesis of terpene compounds and to explain their regulation. Up to now, two TPS genes were identified and functionally characterized in different *T. caespititius* genotypes, a γ-terpinene and an α-terpineol synthase (Lima et al. 2013). The enzymes encoded by these two genes were involved in the synthesis of the main components, carvacrol, thymol and α-terpineol, identified in the thymus essential oils (Paulose and Croteau 1978; Dewick 2002; Johnson et al. 2004; Crocoll et al. 2010; Crocoll 2011).

1.2. Secondary metabolism

Plants produce a vast and diverse range of organic compounds, the great majority of which does not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites, are distributed among limited taxonomic groups within the plant kingdom (Croteau et al. 2000, Taiz and Zeiger 2010). These compounds were thought to be simply functionless end products of metabolism, or metabolic wastes (Taiz and Zeiger 2010), however their functions, many of which remain unknown, are being elucidated (Croteau et al. 2000; Taiz and Zeiger 2010).

The study of these compounds was pioneered by organic chemists of the 19th and early 20th centuries who were interested in these substances due to their importance as medicinal drugs, poisons, flavors, and industrial materials. Recently, many secondary metabolites have been suggested to have important ecological functions in plants, such as protection against herbivory and microbial pathogens, attraction of pollinators and seed dispersing animals and as agents of plant-plant

competition (Taiz and Zeiger 2010).

Plants are a rich source of secondary metabolites that have medicinal and aromatic properties. According to some authors, at least ≈100,000 of secondary metabolites are known to occur in 50,000 plant species and ≈4,000 new secondary metabolites are being discovered every year from a variety of plant species (Verpoorte et al. 1999; Gomez-Galera et al. 2007). For thousands of years, these natural plant products have been utilized in the form of drugs, antioxidants, flavors, fragrances, dyes, and insecticides (Kumar and Gupta 2008).

More than 1% of these metabolites are lipophilic molecules with low boiling points and high vapor pressures at ambient temperature. They are mainly represented by terpenoids, phenylpropanoids, benzenoids, fatty acid derivatives and amino acid derivatives. These volatile compounds are released from leaves, flowers and fruits into the atmosphere and from roots into the soil. The primary functions of volatiles are to defend plants against herbivores and pathogens, to attract pollinators, seed dispersers, and other beneficial animals and microorganisms, and to serve as signals in plant–plant interaction (Dudareva and Pichersky 2008).

1.2.1. Terpenes

More than 65,000 isoprenoid compounds (terpenes) are produced naturally (Koksal et al. 2011; Oldfield and Lin 2012). These molecules represent the oldest and the largest class of small biomolecules, constituting the so-called “terpenome”. They perform a large variety of functions and properties in all living organisms, making them an incredible resource of natural products (Chang and Keasling 2006). They are widely represented in plants, where they act as insecticides, pesticides, pollinator attractors, hormones, flavors, pigments, etc. Their features make terpenes attractive for applications in food, and cosmetics (for example as essential oils), and chemical, pharmaceutical and rubber industries (Dudareva et al. 2006; Berthelot et al. 2012).

All terpenoids are synthesized from the universal five carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are derived from two alternate biosynthetic pathways (Fig. 2), plastidic methylerythritol pathway (MEP) and the cytosolic mevalonate pathway (MAV) (Nagegowda 2010). While DMAPP formed in plastids is used by isoprene synthase to form isoprene in some plants. IPP and DMAPP precursors are further condensed by prenyl diphosphate synthases in the respective compartments to form prenyl diphosphate intermediates serving as substrates for a large group of terpene synthase (TPS) enzymes, resulting in the final terpenoid compounds (Dudareva et al. 2006). DMAPP and IPP are fused by prenyltransferases to form geranyl diphosphate (GPP, C10), the usual precursor of the monoterpenes, and DMAPP and two

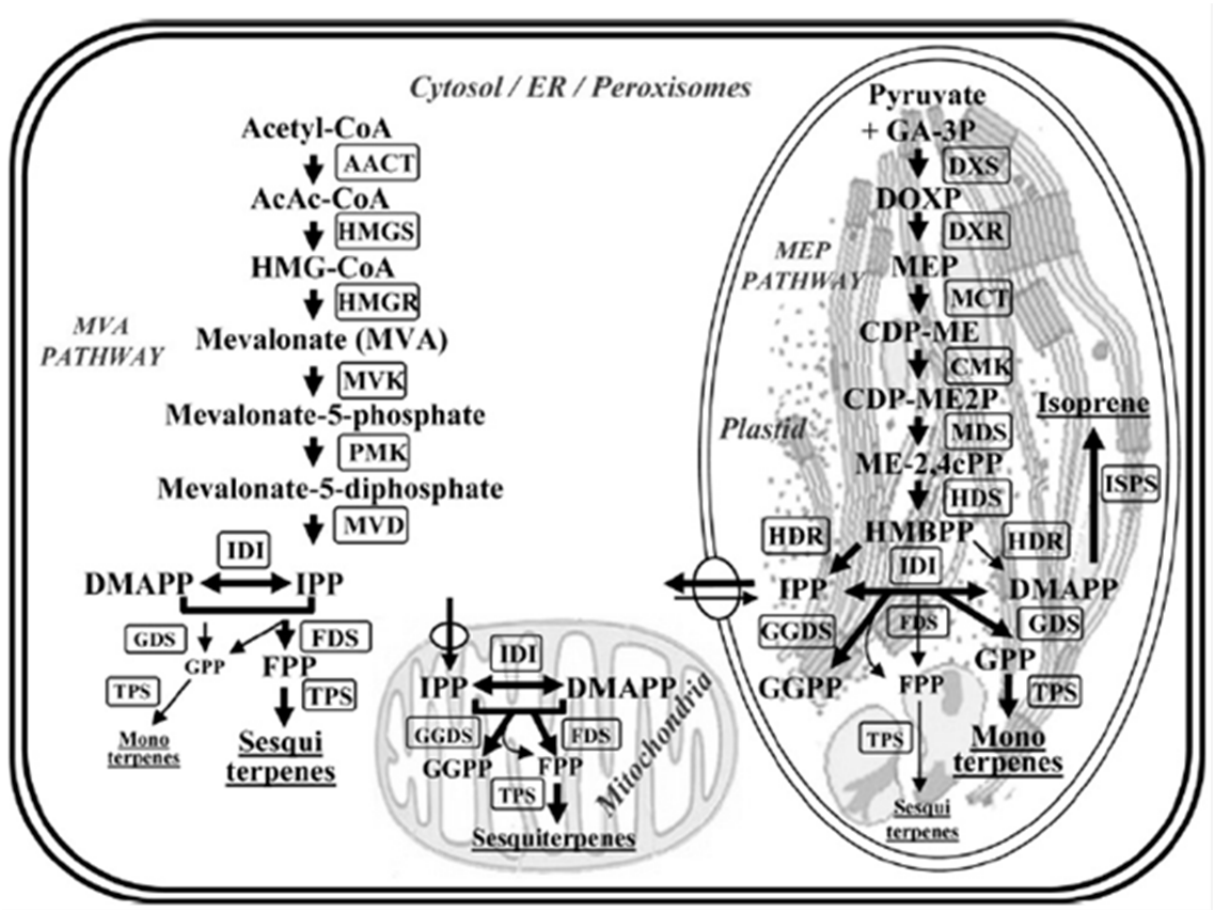

Figure 2. Biosynthetic pathways and their compartmentalization leading to volatile terpenoids in plants. The mevalonic acid (MVA) pathway is located in the cytosol in peroxisomes and in the endoplasmatic reticulum (ER). It starts with three units of acetyl-CoA, and the final product farnesyl pyrophosphate (FPP) is the precursor molecule for all sesquiterpenes. The methyl-erythritol-phosphate (MEP) pathway is located in the plastids and the initial substrates are glyceraldehyde-3-phosphate (GA3P) and pyruvate. Geranyl diphosphate (GPP) is the precursor for all monoterpenes and geranyl geranyl diphosphate (GGPP) the precursor for diterpenes. DMAPP (dimethylallyl diphosphate) is the backbone to which different numbers of the isomer IPP (isopentenyl diphosphate) are added to form GPP, FPP or GGPP. (Adapted from Nagegowda 2010).

units of IPP are fused to form farnesyl diphosphate (FPP, C15) the precursor of most sesquiterpenes. The addition of yet another molecule of IPP originates the C20 compound geranylgeranyl diphosphate (GGPP), the precursor of the diterpenes. Finally, FPP and GGPP can dimerize yielding the triterpenes (C30) and the tetraterpenes (C40), respectively (Taiz and Zeiger 2010). Thereafter, the linear carbon skeletons of GPP and FPP are converted to the basic terpene skeletons by terpene synthases, a widespread class of enzymes responsible for the huge structural diversity of mono- and sesquiterpenes (Tholl 2006; Degenhardt et al. 2009, Nagegowda 2010; Crocoll 2011).

Later modifications (made by cytochrome P450 dependent monooxygenases and various transferases) to the basic parent skeletons produced by the terpene synthases are responsible for generating numerous different terpenoids in plants (Daviet and Schalk 2010; Crocoll 2011). These

secondary transformations most commonly involve oxidation, reduction, isomerization, and conjugation reactions, which impart functional properties to the terpenoid molecules (Croteau et al. 2000; Sangwan et al. 2001; Daviet and Schalk 2010; Crocoll 2011).

1.2.2. Phenylpropanoids

Plants produce a large variety of secondary products that contain a phenol group (a hydroxyl functional group on an aromatic ring) (Croteau et al. 2000). These substances are classified as phenolic compounds. Plant phenolics are a chemically heterogeneous group of approximately 10,000 compounds (Taiz and Zeiger 2010).

Most plant phenolics are products of phenylpropanoid metabolism, which are derived from phenylalanine and tyrosine (Croteau et al. 2000). Phenylpropanoids are not common constituents of plant essential oils, but essential oils of certain species contain abundant or significant proportions of such compounds. The main phenylpropanoids, which have been identified in the oil of certain grass species and chemotypes are eugenol, myristicin, elemicin, chavicol, dillapiole, among others (Sangwan et al. 2001). The phenylpropene skeletal compounds are derived from phenylalanine, synthesized via the shikimic acid pathway (Fig. 3).

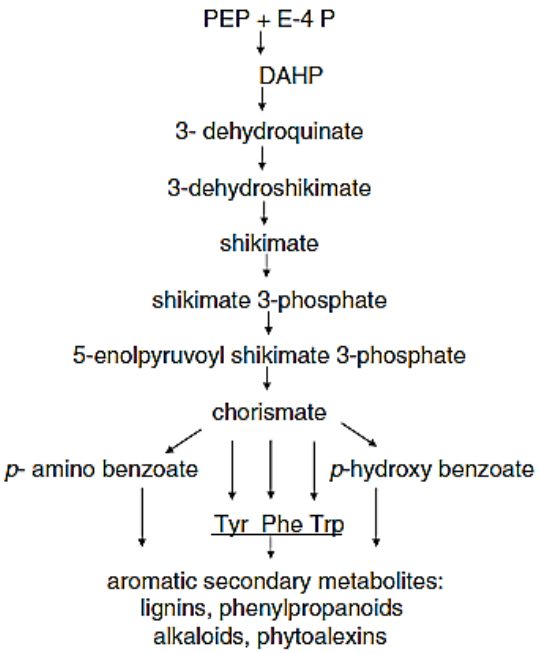

Figure 3. Phenolic compounds are biosynthesized by several different ways. In higher plants, most phenolics are derived from phenylalanine, a product of the shikimic acid pathway. DAHP (3-deoxy-D-arabinoheptulosonate 7-phosphate synthase), E-4 P (erythrose-4-phosphate), PEP (phosphoenol pyruvate), Phe (phenylalanine), Trp (tryptophan), Tyr (tyrosine). (Adapted from Kumar and Gupta 2008).

The shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis (phosphoenol pyruvate) and the pentose phosphate (erythrose-4-phosphate) pathway to chorismate, the biogenetic precursor of the aromatic amino acids, phenylalanine, tyrosine and tryptophan (Croteau et al. 2000; Sangwan et al. 2001; Kumar and Gupta 2008; Taiz and Zeiger 2010). Chorismate is largely confined to chloroplasts and other types of plastids. The synthesis of all intermediates downstream from phenylalanine, takes place in both cytosol and endoplasmic reticulum (Mustafa and Verpoorte 2005). Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine into *trans*-cinnamic acid. Cinnamic acid is then converted to *p*-coumarate by the catalytic action of cinnamate-4-hydroxylase. Coumarate may arise directly from tyrosine under the catalytic action of TAL, tyrosine ammonia lyase (Sangwan et al. 2001; Taiz and Zeiger 2010).

The phenylpropanoids present in the essential oil are derived from phenylalanine and to a minor extent from tyrosine (Sangwan et al. 2001).

Phenolics play a variety of roles in the plant, many serve as defense compounds against herbivores and pathogens, others function in mechanical support, in attracting pollinators and fruit dispersers, in absorbing harmful ultraviolet radiation, or in reducing the growth of nearby competing plants (Croteau et al. 2000; Taiz and Zeiger 2010). The adaptive value of the toxic (i.e. defensive) properties of the phenylpropanoids are likely responsible for the widespread distribution of these chemicals among the Angiosperms, and humans have made extensive use of these properties to further protect their plants and food stocks (Gang et al. 2001).

1.3. Terpene synthases

Terpenoids are major contributors to the chemical arsenal of plants, and they are implicated in the defense against enemies, the attraction of pollinators, and signaling to other plants, (Ehrich and Raven 1964; Harborne 1993). The chemical profile of terpenoids produced by an organism has to evolve and adapt rapidly to stress conditions, what has been called a co-evolutionary arms race (Ehrich and Raven 1964; Harborne 1993). Thus, the basic understanding of the biochemical pathways and the identification of the genes and enzymes involved in the synthesis of terpene compounds is essential. In the last years the interest in these questions combined with major technical advances have led to an increase in the number of plant volatiles identified, as well as to a remarkable progress in discovering the genes and enzymes of volatile biosynthesis (Dudareva and Pichersky 2008).

Terpenes are synthesized by a special class of enzymes, following the formation of the prenyl diphosphate precursors GPP and FPP, a variety of structurally diverse cyclic and acyclic monoterpenes and sesquiterpenes is generated by the action of a large family of enzymes, known as

terpene synthases/cyclases (Trapp and Croteau 2001, Dudareva et al. 2006, Nagegowda 2010). TPS gene family in plant genomes examined range in size; the *Physcomitrella patens* genome contains four TPS genes (Rensing et al. 2008) while 152 were identified in *Vitis vinifera* (Martin et al. 2010; Chen et al. 2011).

Terpene synthases can be divided into three functional classes, namely monoterpene synthases, sesquiterpene synthases, and diterpene synthases (Fig. 4), and are approximately 550–860 amino acids long and 50–100 kDa in size (Bohlmann et al. 1998, Keeling and Bohlmann 2006). Sesquiterpene synthases are usually smaller than the others enzymes, due to absence of the N-terminal signal peptide that targets the initial translation product towards the plastids (50-70 amino acids). The diterpenes are approximately 210 amino acids (aa) longer than monoterpene synthases because of an additional conserved sequence present in the N-terminal domain (Bohlmann et al. 1998). Angiosperm terpene synthases contain 6 introns and 7 exons (Trapp and Croteau 2001).

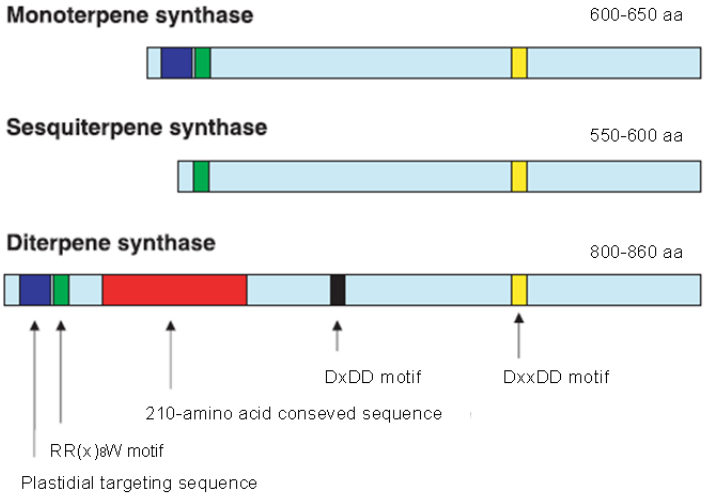

Figure 4. Illustrative scheme of terpene synthase enzymes. (Adapted from Keeling and Bohlmann 2006).

Tertiary structure of TPSs consist entirely of α-helices with short connecting loops and turns and are organized into two structural domains, N-terminal and C-terminal. The C-terminal contains the active site (Degenhardt et al. 2009). The protein active site is a hydrophobic pocket formed by six α-helices and closed to the outside by two loops which are located on the protein surface (Degenhardt et al. 2009).

Almost all terpene synthases contain the aspartate-rich DDxxD motif, which is involved in the coordination of divalent metal ions (Mg^{2+} or Mn^{2+}) for substrate binding (Degenhardt et al. 2009; Crocoll 2011). Direct-mutations in this domain resulted in a decrease of enzyme activity and synthesis of abnormal products, demonstrating the importance of this domain for proper enzyme function (Cane et al. 1996; Little and Croteau 2002; Seemann et al. 2002). The arginine-rich $RR(x)_8W$ motif is located

in the N-terminus, after the transit peptide, and appears to be essential for cyclization of GPP (Fig. 5), and for the enzymatic activity of many monoterpene synthases (Williams et al. 1998; Crocoll 2011; Demissie et al. 2011). LQLYEASFLL (Wise et al. 1998) and (N,D)D(L,I,V)x(S,T)xxxE (Roeder et al. 2007; Degenhardt et al. 2009) are two partially conserved sequences that play roles in catalysis and second metal ion binding, respectively.

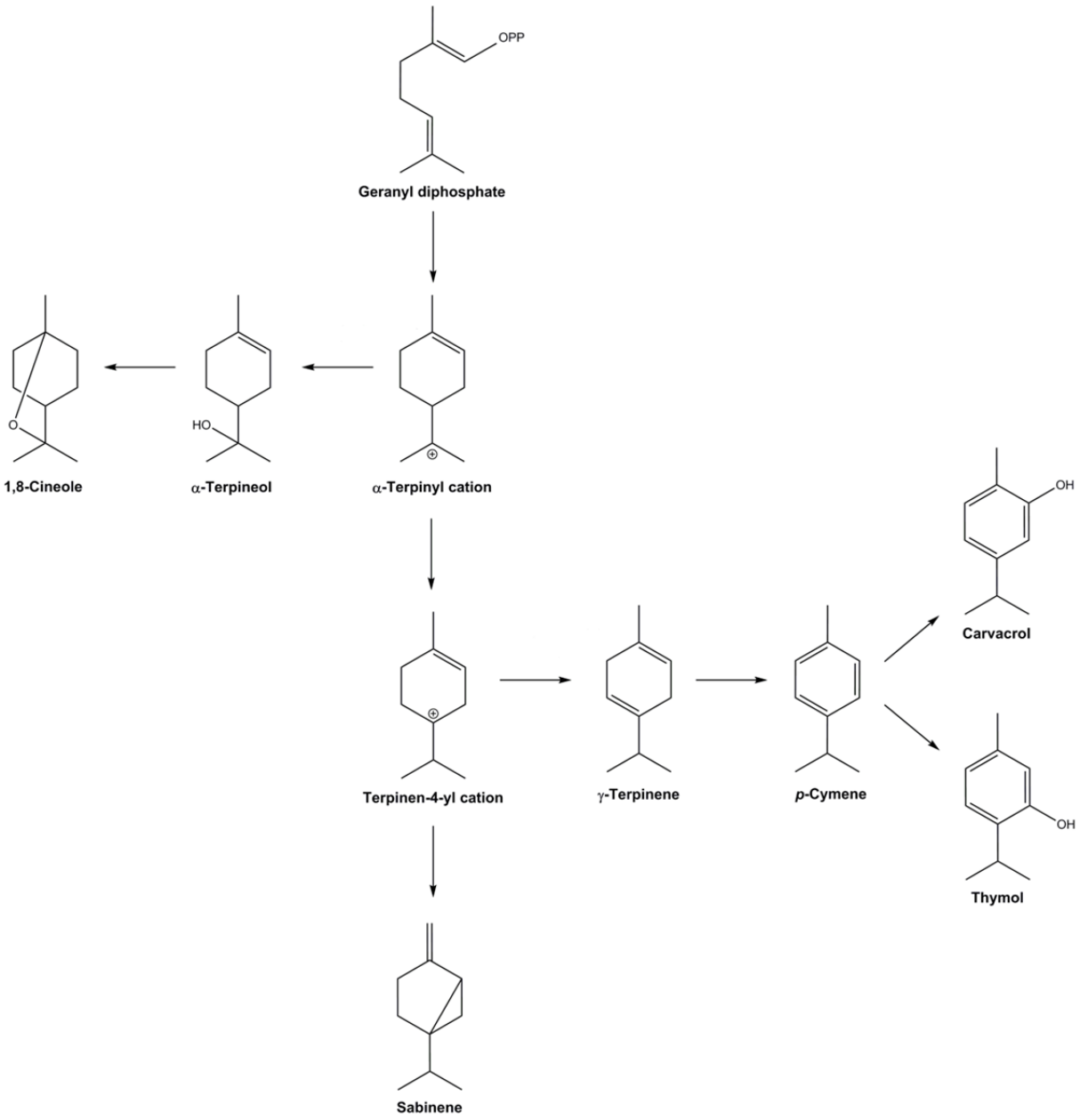

Figure 5. Reaction mechanisms of monoterpene formation from geranyl diphosphate (GPP) as substrate. (Adapted from Galata 2013).

Most plant terpene synthases have the ability to synthesize multiple products from a single substrate with high regio- and stereospecificity (Tholl 2006; Degenhardt et al. 2009). A suggested reason for this is that some of the minor products formed may have been derived from reaction

intermediates which suffered early termination during their metabolic pathway (Degenhardt et al. 2009). Another possible explanation is that multi-product formation could result from an evolutionary adaptation of TPSs to produce the greatest number of products using the least amount of genetic and enzymatic machinery as possible. Active site conformation flexibility of the TPSs has also been proposed as a factor contributing to multi-product formation from a single TPS (Degenhardt et al. 2009).

Previous phylogenetic analyses divided the terpene synthase gene family into seven subfamilies (TPSa through TPSg) based on sequence relatedness, functional assessment and gene architecture (Bohlmann et al. 1998; Trapp and Croteau 2001; Martin et al. 2004). TPSa subfamily consists mostly of sesquiterpene and diterpene synthases from angiosperms. TPSd comprise mono-, sesqui- and di-terpene synthases from gymnosperm. Many monoterpene synthases, including identified monoterpene synthases from Lamiaceae, belong to the TPSb subfamily (Bohlmann et al. 1998; Trapp and Croteau 2001). TPSc, TPSe and TPSf are represented by single angiosperm terpene synthases, copalyl diphosphate synthase, kaurene synthase and linalool synthase, respectively. Linalool synthase from *Arabidopsis* along with myrcene and (*E*)-β-ocimene synthases from snapdragon and strawberry nerolidol synthase belong to TPSg subfamily (Dudareva et al. 2003; Dudareva et al. 2006). More recently, the analysis was extended to include new TPS sequences and also recognizes seven TPS subfamilies, the original TPSa, TPSb, TPSc, TPSd, and TPSg, a merged clade of the original TPSe and TPSf subfamilies, now designated as TPSe/f, and a new subfamily TPSh (Chen et al. 2011). This new clade is represented by eight *Selaginella moellendorffii* TPSs (Chen et al. 2011). All the members within these classes share at least 40% similarity (Bohlmann et al. 1998).

Hemi-, mono-, sesqui-, and diterpene synthases are evolutionarily related to each other (Tholl 2006). All monoterpene synthases share a common carbocation mechanism of action, which begins with a metal ion-dependent ionization of the substrate, yielding a cationic intermediate. This intermediate then undergoes a series of rearrangements including cyclizations or hydride shifts until there is proton loss or a nucleophile is added (Degenhardt et al. 2009). As mentioned above, terpene synthase products are frequently further changed at the primary terpene skeletons by hydroxylation, dehydrogenation, acylation and other reaction types. The cytochrome P450 monooxygenases and oxidoreductases play an important role in these downstream modifications (Dudareva et al. 2004, Crocoll 2011).

Research on TPS has concentrated on a few species with economic importance or designated as model taxa, however many more terpene synthases remain to be described (Degenhardt et al. 2009). Plant enzymes relating to the biosynthesis of oil compounds, particularly mono- and sesquiterpene synthases, have been investigated in Lamiaceae plants, such as *Lavandula* spp., *Mentha* spp.,

Ocimum basilicum, *Origanum vulgare*, *Perilla* spp., *Salvia* spp. and *Thymus* spp. (Table 2). In what concerns *Thymus caespititius*, as mentioned previously, two monoterpene synthases were recently characterized (Lima et al. 2013).

Table 2. Terpene synthases identified to date in Lamiaceae family.

Species	Function (based on main product(s))	GenBank accession no.	References
Agastache rugosa	(+)-Limonene synthase (ArLMS)	AY055214	Maruyama et al. 2002
Isodon eriocalyx	Copalyl diphosphate synthase (CPS1)	HQ455833	Li et al. 2012
Lavandula angustifolia	*trans*-α-Bergamotene synthase (LaBERS)	DQ263742	Landmann et al. 2007
	(+)-Limonene synthase (LaLIMS)	DQ263740	
	(-)-Linalool synthase (LaLINS)	DQ263741	
	β-Phellandrene synthase (LabPHLS)	HQ404305	Demissie et al. 2011
	1,8-Cineole synthase (LaCINS)	JN701461	Demissie et al. 2012
	τ-Cadinol synthase (LaCADS)	JX401282	Jullien et al. 2014
	E-β-Caryophyllene synthase (LaCARS)	JX401283	
	Germacrene D synthase (LaGERDS)	JX401284	
Lavandula x intermedia	1,8-Cineole synthase (LiCINS)	JN701459	Demissie et al. 2012
	9-epi-Caryophyllene synthase (LiCPS)	KF470962	Sarker et al. 2013
Lavandula latifolia	1,8-Cineole synthase (LlCINS)	JN701460	Demissie et al. 2012
	Linalool synthase	DQ421801	Tan et al. (unpublished; only available on Genbank)
Lavandula pedunculata subsp. *lusitanica*	α-Fenchol synthase	JX501511	Benabdelkader et al. (unpublished; only available on Genbank)
	Germacrene A	JX501513	
	α-Pinene synthase	JX501512	
Lavandula stoechas	α-Fenchol synthase	JX501514	Benabdelkader et al. (unpublished; only available on Genbank)
	Germacrene A	JX501516	
	α-Pinene synthase	JX501515	
Lavandula viridis	α-Fenchol synthase	JX501517	Benabdelkader et al. (unpublished; only available on Genbank)
	Germacrene A	JX501519	
	α-Pinene synthase	JX501518	
Mentha arvensis	(*E*)-β-Farnesene synthase	FJ999995	Bhattacharya and Verma (unpublished; only available on Genbank)
	Limonene synthase	JX555963	Wang et al. 2013
Mentha canadensis	Limonene synthase	JX555966	Wang et al. 2013
Mentha cardiaca	Limonene synthase	JX555964	Wang et al. 2013
Mentha citrata	(-)-Linalool synthase	AY083653	Crowell et al. 2002
Mentha longifolia	Limonene synthase	AF175323	Crock and Croteau (unpublished; only available on Genbank)
Mentha x piperita	*E*-β-Farnesene synthase	AF024615	Crock et al. 1997
	E-β-Farnesene synthase (tpsa11)	AJ786642	Prosser et al. 2006
	cis-Muuroladiene synthase (MxpSS1)	AJ786641	
	Limonene synthase	EU108697	Gupta et al. (unpublished; only available on Genbank)
Mentha spicata	(-)-Limonene synthase (pLC5.2)	L13459	Colby et al. 1993

(Cont.)

Species	Function (based on main product(s))	GenBank accession no.	References
Mentha suaveolens	Limonene synthase	JX555970	Wang et al. 2013
Ocimum basilicum	(-)-endo-Fenchol synthase (FES)	AY693648	Iijima et al. 2004a
	(+)-Linalool synthase (LIS)	AY693647	
	Terpinolene synthase (TES)	AY693650	
	γ-Cadinene synthase (CDS)	AY693645	Iijima et al. 2004b
	Geraniol synthase (GES)	AY362553	
	Germacrene-D synthase (GDS)	AY693644	
	β-Myrcene synthase (MYS)	AY693649	
	α/β-Selinene synthase (SES)	AY693643	
	α-Zingiberene synthase (ZIS)	AY693646	
Origanum majorana	Monoterpene synthase	KC432651	Crocoll et al. (unpublished; only available on Genbank)
Origanum vulgare	Bicyclogermacrene synthase (OvTPS4)	GU385973	Crocoll et al. 2010
	E-β-Caryophyllene synthase (OvTPS6)	GU385970	
	(-)-Germacrene-D synthase (OvTPS3)	GU385976	
	trans -β-Ocimene synthase (OvTPS7)	GU385967	
	Sabinene synthase (OvTPS1)	GU385980	
	γ-Terpinene synthase (OvTPS2)	GU385978	
Perilla citridora	Limonene synthase	AF233894	Ito et al. 2000
	Geraniol synthase (PcTps-C)	DQ088667	Ito and Honda 2007
Perilla frutescens	(-)-Limonene synthase (PFLC1)	AAG31438	Yuba et al. 1996
	Myrcene synthase (PTS-5526)	AF271259	Hosoi et al. 2004
	Geraniol synthase (PfTPS-PL)	DQ234300	Ito and Honda 2007
Perilla hirtella	Geraniol synthase (Tps-5073G30)	FJ644547	Masumoto et al. 2010
	Linalool synthase (Tps-5042L13)	FJ644548	
Perilla setoyensis	Geraniol synthase (Tps-5031G8)	FJ644545	Masumoto et al. 2010
	(-)-Linalool synthase (Tps-5031L19)	FJ644544	
Plectranthus barbatus	ent-Kaurene synthase	KC702394	Zerbe et al. 2013
Pogostemon cabli	γ-Curcumene synthase (PatTpsA)	AY508726	Deguerry et al. 2006
	(+)-Germacrene-A synthase (PatTpsCF2)	AY508728	
	(-)-Germacrene-D synthase (PatTpsBF2 and PaTpsB15)	AY508729	
	(-)-Pathoulolol synthase (PatTps177)	AY508730	
	Patchoulol synthase	KF983531	Hartwing et al. 2014
Rosmarinus officinalis	1,8-Cineole synthase	JX050194	Rudolph et al. (unpublished; only available on Genbank)
	1,8-Cineole synthase	DQ839411	Tan et al. (unpublished; only available on Genbank)
	Limonene synthase	DQ421800	
	Pinene synthase	EF495245	
	Copalyl diphosphate synthase (RoCPS1)	KF805857	Brückner et al. 2014
	Kaurene synthase-like (RoKSL1/2)	KF805858(9)	
Salvia fruticosa	1,8-Cineole synthase (Sf-CinS1)	DQ785793	Kampranis et al. 2007
Salvia miltiorrhiza	Geranylgeranyl diphosphate synthase (SmGGPP)	FJ643617	Kai et al. 2010
	Copalyl diphosphate synthase (SmCPS)	JX156302	Yang et al. 2013
	Kaurene synthase (SmKSL)	EF635966	

(Cont.)

Species	Function (based on main product(s))	GenBank accession no.	References
Salvia officinalis	(+)-Bornyl diphosphate synthase (SBS)	AF051900	Wise et al. 1998
	1,8-Cineole synthase (SCS)	AF051899	
	(+)-Sabinene synthase (SSS)	AF051901	
Salvia pomifera	Sabinene synthase (Sp-SabS1)	DQ785794	Kampranis et al. 2007
Salvia sclarea	Hydroxy-copalyl diphosphate synthase	HQ641451	Günnewich et al. 2013
Salvia stenophylla	(+)-3-Carene synthase	AF527416	Hölscher et al. 2003
Schizonepeta tenuifolia	(+)-Limonene synthase (dLMS)	AF282875	Maruyama et al. 2001
Thymus caespititius	γ-Terpinene synthase (Tctps2)	KC181099	Lima et al. 2013
	α-Terpineol synthase (Tctps5)	KC181102	
Thymus x citriodorus	γ-Terpinene synthase	KF494252	Rudolph et al. (unpublished; only available on Genbank)
Thymus serpyllum	γ-Terpinene synthase	KF494251	Rudolph et al. (unpublished; only available on Genbank)
Thymus vulgaris	(*E*)-Sabinene hydrate (TPS6)	JX946357	Krause et al. 2013
	(*Z*)-Sabinene hydrate (TPS7)	JX946358	
	Linalool synthase (TPS3/TPS4)	JX997982(3)	Schimmel et al. (unpublished; only available on Genbank)
	γ-Terpinene synthase (TPS2)	JX997981	
	α-Terpineol synthase (TPS5)	KC461937	
	γ-Terpinene synthase	JQ957864	Rudolph et al. (unpublished; only available on Genbank)

1.4. Regulation of the terpene biosynthesis

The biosynthesis of terpenes is mediated by terpene synthases and regulated by environmental and developmental factors (Tholl 2006). Plant volatiles are spatially and temporally regulated, they are biosynthesized and emitted from specific plant tissues at a particular time, indicating that volatile terpenoid biosynthesis is mainly regulated at the transcription level (Dudareva et al. 2006; Nagegowda 2010). The synthesis of volatiles increases during the early stages of organ development and then either remains relatively constant or decrease over the organs life period (Bouwmeester et al. 1998; Dudareva and Pichersky 2000; Gershenzon et al. 2000).

Many features of plant development and metabolism are light regulated. The release of volatile terpenes displays a rhythmic pattern with the maximum emission during the day or night, generally overlapping the activity of related insects, herbivores (Dudareva et al. 2004, Cheng et al. 2007). The rhythmic and developmental regulation of terpenes has been reported in different species (Aharoni et al. 2003; Dudareva et al. 2003; Lu et al. 2002). For example, in *Artemisia annua*, the synthesis of β-pinene fluctuates with the day-night rhythm and is generally higher during the day than in the night (Lu et al. 2002).

Many terpenoids are specifically induced in response to elicitor or herbivores damage. This type of

defense has been studied in many species (Paré and Tumlinson 1997; Van Poecke et al. 2001; Rodriguez-Saona et al. 2003). Two-spotted spider mites, *Tetranychus urticae*, induced emission of (*E*)-β-ocimene and transcript accumulation of (*E*)-β-ocimene synthase in *Lotus japonicus* (Arimura et al. 2004). In *Arabidopsis*, caterpillars (*Pieris rapae*) induced the myrcene/ocimene synthase and the β-ocimene synthase, leading to increased myrcene (Van Poecke et al. 2001). The transcript level of (+)-δ-cadinene synthase was induced in cotton stems (Alchanati et al. 1998) and suspension cultured cells (Liu et al. 1999) after treatment with fugal elicitors from *Verticillium dahlia*. Artificial wounding or jasmonic acid (JA) treatments are often used to mimic herbivore and pathogen attacks to induce terpene synthases (Van Schie et al. 2007; Zulak et al. 2009). The volatile emissions, oleoresin components and terpene synthase transcript levels in *Sitka spruce* were analyzed upon treatment with methyl jasmonate (MeJa). The treatment induced traumatic resin accumulation in stems and up-regulation of several terpene synthase transcripts (Miller et al. 2005). The JA induced the expression of linalool synthase in tomato (*Lycopersicon esculentum*) trichomes, contributing for the linalool increase in these plants (Van Schie et al. 2007).

Abiotic factors such as light intensity, CO_2 concentration, temperature, humidity and nutrients can significantly influence the volatiles synthesis (Gershenzon et al. 2000; Gouinguene and Turlings 2002). UV-B radiation increased the peppermint biomass and the quantity of volatiles oils. However the composition of the oils was not changed (Maffei and Scannerini 2000). mRNA levels of a multifunctional terpenoid synthase was increased with salt concentration in roots and leaves of *Kandelia candel* (Basyuni et al. 2009).

2. The project

2.1. Timeliness, relevance, originality and goals

The project, which will be described in the following chapters, was designed to identify terpene synthase genes that are responsible for *T. caespititius* essential oils and also to try to understand how those genes are regulated.

As became evident in the previous section, great progress has been made in elucidating plant terpene biosynthetic pathways at the gene and enzyme levels. Since the first descriptions of terpene synthase genes, research efforts focused on identification of new TPS genes in economically important plants, on functional characterization, regulation and recently on metabolic engineering. The manipulation of plant terpene metabolism has long been a focus of interest in plant biotechnology. An improved knowledge on plant terpene metabolism will help the manipulation of the terpene pathway

for changing agronomic traits such as fruit flavors (Lewinsohn et al. 2001; Davidovich-Rikanati et al. 2007), floral scent (Lücker et al. 2001), plant defense against pests (Schnee et al. 2006) and high level production of known and novel biochemical (Bohlmann and Keeling 2008; Kirby and Keasling 2009).

The existing distinct *T. caespititius* chemotypes, namely thymol, carvacrol, α-terpineol, sabinene, and the mixed chemotypes, thymol/carvacrol and thymol/sabinene/carvacrol resulted from natural evolution. So, all thyme plants probably have the genetic potential to synthesize most of the compounds found in this species. Understanding how *T. caespititius* terpene synthase genes are regulated and which parameters affect their expression will be helpful to elucidate the chemical diversity of the essential oils. Moreover understanding which factors are significant for high accumulation of commercially important metabolites (e.g. carvacrol, thymol) may allow us to control how and when they accumulate.

Giving the interest on this species' essential oils, the main goals of this project were:

(i) To establish *T. caespititius in vitro* cultures, from plants with different chemotypes, and evaluate the stability of the essential oil composition as compared with the field-grown plants;

(ii) To isolate and characterize putative TPSs genes from the *T. caespititius* plants, eventually responsible for quantitative variations in the essential oils composition;

(iii) To evaluate how the expression of TPSs genes is regulated by different stimuli, using the *in vitro* established *T. caespititius*.

2.2. The project: chapter by chapter

In order to study the essential oil production in *Thymus caespititius* plants belonging to different chemotypes, it was necessary to plant the accessions collected from Azores Islands in an easily accessible place. The plants were cultivated at ESAC (Escola Superior Agrária de Coimbra), in Coimbra (Portugal).

In **Chapter II**, selected *T. caespititius* genotypes (chemotypes) were established *in vitro*, in order to avoid plant collection from their field plots, and to fully control the environmental growth conditions, while ensuring the production of enough biomass for the different tasks. Despite the difficulties to establish the shoot cultures, proliferative shoots were obtained. The essential oils produced from the *in vitro* genotypes were evaluated for yield and chemical composition in the proliferating shoot cultures and compared to those from field-grown plants.

The monoterpene biosynthesis in *T. caespititius* are described and discussed in **Chapter III**. The identification and characterization of terpene synthase genes was first conducted in field-grown plants (Lima et al. 2013). In **Chapter III**, the experiments, there described, were performed in proliferative

shoot cultures with the aim to identify and characterize TPS genes responsible for the chemical diversity observed in *T. caespititius* essential oils. Also, TPS transcripts levels were evaluated during the shoot development to determine at which stage there was higher transcripts accumulation.

The results obtained from the experiments described in **Chapter II** and **Chapter III** were crucial for the following steps of the project. Until this point the *T. caespititius* cultures were studied in an identical way, but from this point onwards, some strategies were applied only for some genotypes.

In **Chapter IV**, the experiments performed aiming to assess the effect of some biotic and abiotic stresses on *T. caespititius* shoots cultures essential oils and on terpene synthase genes, and also to understand the role of the *Tctps* genes in essential oil composition. Nutrient composition (abiotic) and *Botrytis cinerea* fungal extracts (biotic) were the selected stress factors applied to shoots *in vitro* culture.

In **Chapter V** the major conclusions of each previous chapter are reviewed. Finally, some future directions on *T. caespititius* essential oils research are proposed.

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Chapter II

Essential oil production in shoot cultures vs field-grown plants of *Thymus caespititius*

Part of the results presented in this chapter were previously published.

Mendes MD, Figueiredo AC, Oliveira MM, Trindade H. **2013**. Essential oil production in shoot cultures *versus* field-grown plants of *Thymus caespititius*. Plant Cell Tiss Organ Cult 113:341-351.

1. Abstract

Thymus caespititius Brot. is an important aromatic species, due to the synthesis and production of essential oils for the pharmaceutical and food industries. In the present study, levels of essential oils from five genotypes, including plants with carvacrol (C), carvacrol/thymol (CT) and sabinene/carvacrol (SC) chemotypes and two seedlings from a mother-plant α-terpineol (G1 and G2) were evaluated in proliferating shoot cultures (6 to 24 subcultures following establishment) and compared to those from field-grown plants. The essential oils were isolated by hydrodistillation and analyzed by GC and GC-MS. Cultures grown under *in vitro* conditions, evaluated over two years, were found to maintain stable essential oils composition, except G *in vitro* shoots. C shoots revealed a chemical profile similar to the field-grown plant, with carvacrol (20-69% in shoots and 71% in the field plants) as the main component of the essential oils. Carvacryl acetate was the second major compound in *in vitro* shoots cultures, and its relative abundance varied (6-40% in shoot cultures and 4% in field-grown plants). For the CT genotype, carvacrol (42%) and thymol (23%) were the main essential oil components detected in field-grown plants; in proliferating shoot cultures carvacrol levels attained 16-28%, while thymol varied from 17-25%, closely followed by carvacryl acetate (11-23%) and thymyl acetate (9-15%). In the SC genotype, carvacrol (11-28%), sabinene (18-49%), and thymol (8-12%) were the main essential oil components detected in both field-grown and proliferating shoot cultures. In the case of the G *in vitro* shoots, they showed a different essential oil profile from the mother-plant and it was impossible to determine a specific chemotype in these plants. Very low levels of α-terpineol were detected in the shoots oils and the inverse was observed for thymol, carvacrol methyl ether, thymyl acetate and carvacrol.

These experiments showed that the essential oil composition in proliferating shoot cultures (resulting from axillar buds) was not only stable, but also qualitatively similar to that of field-grown plants, notwithstanding minor quantitative differences.

2. Introduction

As mentioned in chapter I, *Thymus caespititius* Brot. is an endemic species from the NW Iberian Peninsula and the Azores and Madeira archipelagos (Santos et al. 2005), showing chemical polymorphism with well-defined carvacrol, thymol, α-terpineol, sabinene, carvacrol/α-terpineol, α-terpineol/τ-cadinol and carvacrol/thymol chemotypes (Salgueiro 2006; Figueiredo et al. 2008a; Trindade et al. 2008). *T. caespititius* essential oils have displayed high levels of antimicrobial (Dandlen et al. 2011), antioxidant (Miguel et al. 2003; Miguel et al. 2004, Dandlen et al. 2010), and nematicidal

(Barbosa et al. 2010, Barbosa et al. 2012; Faria et al. 2013) activities.

Thymus spp. can be propagated sexually or asexually. Reproduction through seeds is usually slow and the plants display substantial variation in a number of characteristics, such as growth rate and essential oil composition. Vegetative propagation techniques are therefore an alternative that makes it possible to clone a genotype of interest (Zuzarte et al. 2010).

Micropropagation is a good method for the multiplication of selected genotypes and chemotypes of several medicinal and aromatic plants (Rout et al. 2000). The *in vitro* cultured plants can subsequently be used for a variety of studies, avoiding collection from their natural habitat. Besides their importance in facilitating plant propagation, *in vitro* techniques provide model systems with which to study the production, accumulation, and metabolism of important compounds.

To date, several reports have been published detailing *in vitro* propagation of *Thymus* spp. from seeds, including *T. hyemalis* (Nordine et al. 2013), *T. lotocephalus* (Coelho et al. 2012), *T. mastichina* (Fraternale et al. 2003a; Fraternale et al. 2003b), *T. piperella* (Sáez et al. 1994), *T. sipyleus* (Erdag and Yurekli 2000) and *T. vulgaris* (Olszowska and Furmanowa 1987; Lê 1989; Tisserat and Vaughn 2001; Affonso et al. 2009; Ozudogru et al. 2011). *In vitro* propagation from field-grown plants through multiplication of nodal segments has been reported for *T. mastichina* (Mendes and Romano 1999), *T. longicaulis* (Ozudogru et al. 2011) and *T. vulgaris* (Furmanowa and Olszowska 1992). Preliminary data on *T. caespititius* micropropagation has already been published (Mendes et al. 2010).

Given the interest of *T. caespititius* essential oils, an *in vitro* proliferation method for *T. caespititius* adult plants with known chemotypes was developed, and essential oil production was compared with that of field-grown plants. The main goal was to define the conditions needed to grow shoots in a controlled environment that provided chemotype stability.

3. Material and Methods

3.1. Plant material

T. caespititius adult plants belonging to several different chemotypes were collected on São Jorge Island (Azores archipelago, Portugal) and on Gerês (North of Portugal) and planted in separate plots (Fig. 1A) at Escola Superior Agrária de Coimbra (Coimbra, Portugal), where they were maintained on small patches in open field. One year later, one plant from the carvacrol/thymol chemotype (CT), one from the sabinene/carvacrol chemotype (SC) and one from α-terpineol chemotype (G) were used to establish *in vitro* shoot cultures and were also used as the reference field-grown plants. It was impossible to establish the G genotype from axillary buds, so from this plant were used seeds. Before the beginning of this project, one plant from carvacrol chemotype (C) was already established *in vitro*

(Mendes et al. 2010).

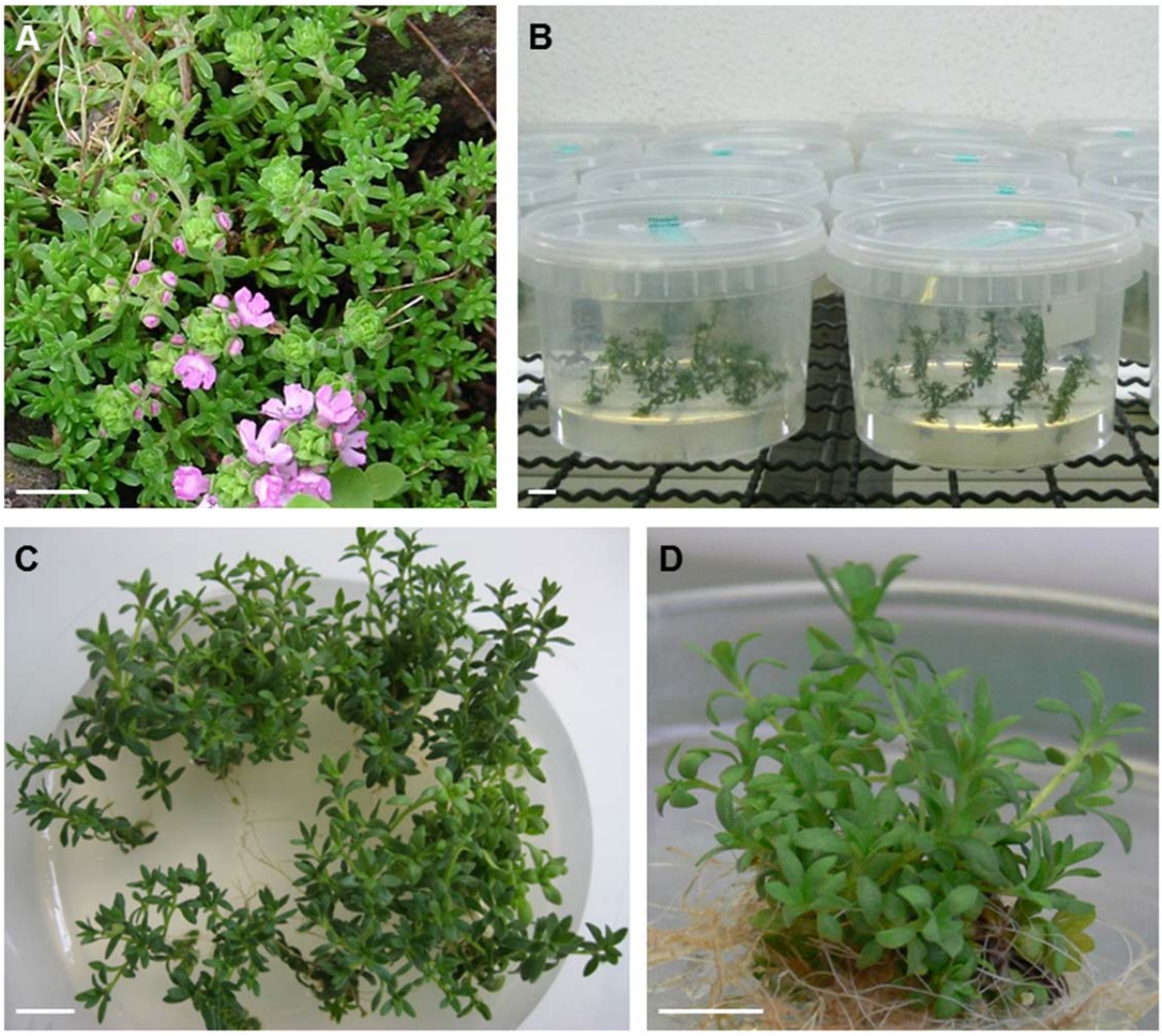

Figure 1. *T. caespititius* field-grown plants (A) and *in vitro* shoot cultures (B-D). B) *In vitro* cultures in the containers. C,D) Detail of the multiple shoots produced by outgrowth of the axillary buds maintained on MS medium with 0.4 $mg.L^{-1}$ BA and 0.1 $mg.L^{-1}$ IBA. D) Detail of spontaneous root formation in shoot cultures. Bars = 1 cm.

3.2. Establishment and maintenance of the *in vitro* shoot cultures

Shoot-tips (1 cm long) from the three *T. caespititius* genotypes, in an adult vegetative state, were separately excised and thoroughly washed with running tap water for 5 min, and then dipped in a commercial detergent solution (10 drops per 100 mL of distilled water) immersed in an ultrasound bath for 15 min. These explants were then surface disinfected in a 50% commercial bleach solution (sodium hypochlorite, 3-5% active chlorine) for 30 min under continuous agitation (90 rpm), and further washed twice in sterile distilled water.

Thirty-six disinfected explants were inoculated on basal MS (Murashige and Skoog 1962) medium

with 3% (w/v) sucrose, supplemented with 1.0 mg.L^{-1} BA (6-benzyladenine) and 0.25 mg.L^{-1} IBA (indole-3-butyric acid). The pH was adjusted to 5.8 prior to the addition of 0.8% (w/v) agar and autoclaved at 121ºC for 15 min.

Seeds of *T. caespititius* (α-terpineol chemotype) were surface disinfected in a 50% (v/v) commercial bleach solution (sodium hypochlorite, 3-5% active chlorine) in an ultrasound bath for 5 min, and further washed twice in sterile distilled water. Seeds were germinated on solid Schulz medium (Schulz 1971) without growth regulators in a growth chamber at 24ºC under a 16 h photoperiod, with light provided by cool fluorescent lamps (32 μE.m^{-2}.s^{-1}). After one week, the seedlings were transferred separately to basal MS medium with 3% (w/v) sucrose, supplemented with 1.0 mg.L^{-1} BA and 0.25 mg.L^{-1} IBA for proliferative shoot proliferation.

Routine culture conditions were as follows: *T. caespititius* shoots were subcultured every month using microboxes from Combiness ® (Belgium) with the green filter (XXL+) on the lid, to facilitate air exchange, and maintained in a growth chamber at 24ºC under a 16 h photoperiod, with light provided by cool fluorescent lamps (32 μE.m^{-2}.s^{-1}). As a regular procedure, each shoot cluster was subdivided into 3-4 smaller clusters and transferred to fresh medium. Approximately six plant clusters were placed in each microbox.

For shoot elongation, after six subcultures following culture initiation, the shoots were transferred to MS medium with growth regulators reduced to 0.4 mg.L^{-1} BA and 0.1 mg.L^{-1} IBA.

3.3. *In vitro* rooting and acclimatization

Rooting process and acclimatization was only performed for G2 genotype. Shoots with at least three nodes (about 2 cm long), harvested at the end of the subculture period were used for rooting (approximately 30). Rooting was induced by dipping the shoots basal ends in a 0.25 mg.mL^{-1} IBA solution for 2 min, following subculture on half-strength MS medium with reduced sucrose [2% (w/v)] and without growth regulators. Cultures were incubated during 5 days in darkness and then were transferred to the same growth conditions described above, for 8 weeks.

3.4. Statistical Analysis

Growth rate was evaluated considering biomass increase and *in vitro* proliferation rates. Biomass increase was calculated on a fresh weight basis (fw), while proliferation rate was assessed by counting the number of shoots at subculture and following a 30-day growth period under previously defined conditions. These experiments were performed one year after culture establishment (12 subcultures). Initial average shoot weight was 80 mg. Mean values ± standard errors (SE) were

obtained from ten independent replicates and the experiments were performed twice. The statistical significance of data obtained from the five genotypes was determined by one-way analysis of variance (ANOVA) for the three sets of data. All statistical analyses were performed using Microsoft Excel 2010.

3.5. Essential oils isolation

Approximately 30 g of aerial parts of field-grown plants and 3-5 g of *in vitro* shoots (sampled at 6^{th}, 10^{th}, 12^{th}, 16^{th}, 20^{th} and 24^{th} subcultures after *in vitro* establishment) were used for analysis. Essential oils were isolated by hydrodistillation for 3 h, at a distillation rate of 3 $mL.min^{-1}$, using a Clevenger-type apparatus according to the European Pharmacopoeia (Council of Europe 2007), and kept at -20ºC until analysis. Two replicates were used per experiment. The data was calculated as mean values of each experimental point.

3.6. Gas Chromatography (GC)

Gas chromatographic analyses were performed using a PerkinElmer Autosystem XL gas chromatograph equipped with two flame ionization detectors (FIDs), a data-handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.]. Oven temperature was programmed for 45-175°C, at 3°$C.min^{-1}$, subsequently at 15°$C.min^{-1}$ up to 300°C, and then held isothermal for 10 min; injector and detector temperatures were 280°C and 300°C respectively; and the carrier gas (hydrogen) was adjusted to a linear velocity of 30 $cm.s^{-1}$. The samples were injected using a split-sampling technique, ratio 1:50. The volume injected was 0.1 µL, of a pentane-oil solution (1:1). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

3.7. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS unit consisted of a PerkinElmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a PerkinElmer Turbomass mass spectrometer (software version 4.1, PerkinElmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 $cm.s^{-1}$; split ratio,

1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identities of the components were assigned by comparing their retention indices, relative to C_9-C_{20} *n*-alkane indices and GC-MS spectra from a home-made library, which was constructed on the basis of analyses of reference oils, laboratory-synthesized components and commercially available standards.

3.8. Scanning Electron Microscopy (SEM)

Leaves and stems from *T. caespititius* field-grown and *in vitro* plants were prepared for SEM as previously reported (Figueiredo and Pais 1994). Plant material was fixed with 1.5% glutaraldehyde (GA) in 0.05 M sodium cacodylate buffer, pH 7.0, for 45 min at room temperature. After 1-2 min at reduced pressure (26 mmHg), the fixative was replaced with 3% GA in 0.1 M sodium cacodylate buffer, pH 7.0, for 2 h at room temperature. The material was thoroughly rinsed in the same buffer and post-fixed with a 1% osmium tetroxide aqueous solution for 2 h at room temperature. After dehydration in a graded acetone series, the material was dried using the critical-point drying method in a Polaron E3500. Dried specimens were sputter-coated with gold in a Polaron E5350, and the observations were made using a JEOL JSM T220 scanning electron microscope at an accelerating voltage of 15-20 kV.

4. Results

4.1. Shoot cultures

Aseptic shoot-tip cultures of *T. caespititius* with high shoot proliferation were established from the four genotypes, although a high percentage of contamination was responsible for the initial low establishment success (approximately 8%) for CT and SC genotypes. For the G genotype the contamination rates was 100%, so instead of using axillary buds this chemotype was established from seedlings (derived from an α-terpineol mother-plant). Approximately three months after *in vitro* initiation, proliferation started and 100% of the established shoots showed proliferation capacity.

According to preliminary studies, six to eight plant clusters revealed to be the suitable number to obtain good proliferative plant material in each microbox. The average cluster weight (60-100 mg) and shoot number (3-6) also revealed to be important for proliferation. So, as regular procedure, plants clusters with these features were used in each subculture period.

Shoot growth was evaluated by fresh weight increase on MS medium supplemented with 0.4 $mg.L^{-1}$ BA and 0.1 $mg.L^{-1}$ IBA. Fresh weight increase varied between 3.1- and 5.5-fold, while the proliferation rate (considered to be the number of new shoots produced in each subculture) varied from 1.7- to 2.9-fold (Table 1). G2 showed the lower growth and proliferative rates; however the

differences were not statistically significant relatively to the C genotype. The rates were higher for the other three genotypes (CT, SC and G1) but the differences among them were not statistically significant (Table 1).

Table 1. Evaluation of shoot proliferation rate in *in vitro T. caespititius* using two different methods – fresh weight increase and shoots number - after a 30 day period on MS medium supplemented with 0.4 mg.L-1 BA and 0.1 mg.L-1 IBA.

Genotype	Mean fresh weight	Mean number of shoots
C	3.3±0.4 a	2.2±0.3 a,c
CT	5.5±0.4 b	2.6±0.3 a,b
SC	4.6±0.3 b	2.5±0.4 a,b
G1	4.5±0.4 b	2.9±0.4 a,b
G2	3.1±0.3 a	1.7±0.2 c

Data indicate mean ± SE (n = 10), and letters that are different within the same column indicate statistical differences (P ≤ 0.05).

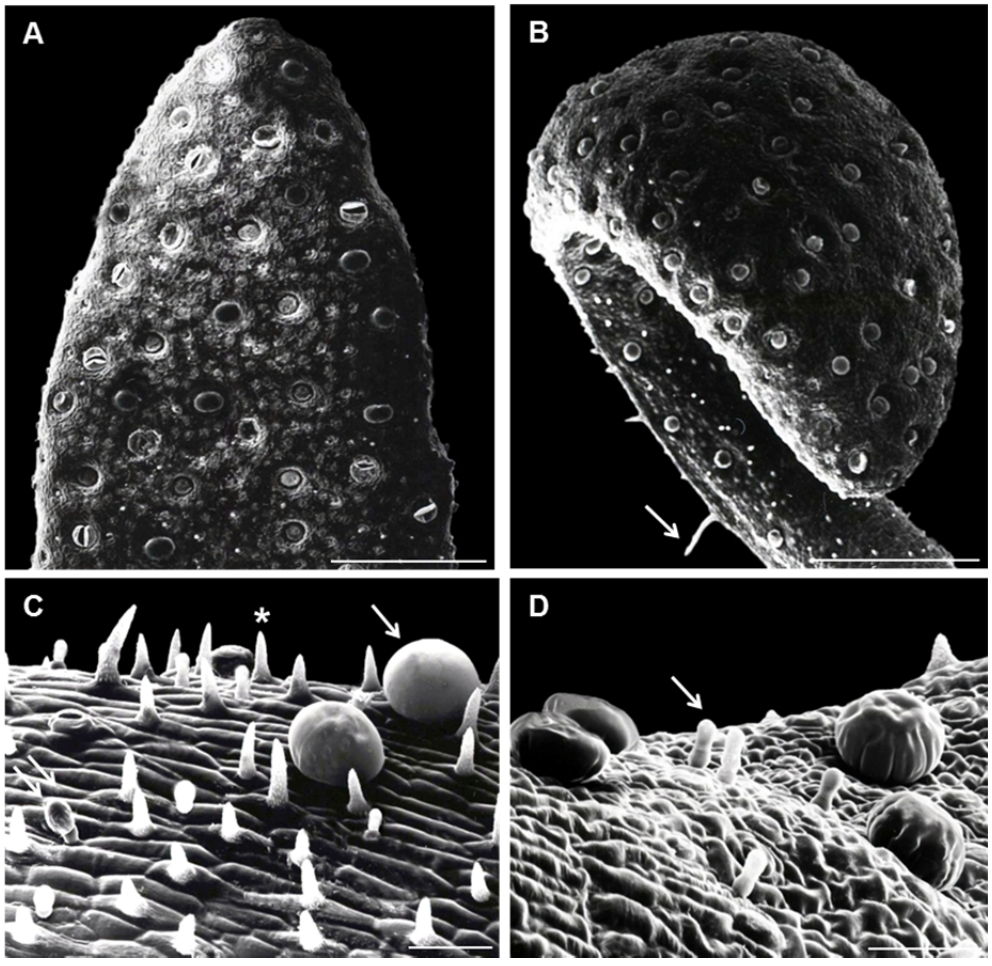

Figure 2. SEM micrographs of *T. caespititius* glandular trichomes from field-grown and *in vitro* shoot cultures. A,B) General aspect of the adaxial surface of a leaf from field-grown and shoot culture, respectively; The nonglandular trichomes were multicellular bent point-shaped-type (B, arrow) and/or conical unicellular (C, star); The glandular trichomes were of two main types: peltate (C, arrow) and capitate, subtype I (C, double arrow) and subtype II (D, arrow). Bars = 500 µm (A, B), 50 µm (C,D).

In vitro grown shoots were morphologically similar to field-grown plants (Fig. 1 and 2), albeit with more elongated internodes. The slightly curled leaves were covered with glandular trichomes with density similar to that of field-grown material (Fig. 2).

In vitro cultures were propagated without *callus* formation. Spontaneous root formation occurred in all genotypes, but these roots were always excised and discarded upon subculture (Fig. 1D).

4.2. Rooting and acclimatization

Rooting percentage was evaluated 8 weeks after G2 shoots were transferred to half strength MS without growth regulators. Only one method was used for rooting induction. This proved to be a reliable method, since it induced rooting without callus formation. Over ≥90% of the shoots rooted with 3 to 5 roots per shoot. Roots were long (approximately 5 cm) and thick.

The acclimatization protocol used for *in vitro* rooted plantlets was successful for all tested plants (approximately 30). During the acclimatization stage, the plantlets continued to grow revealing a successful establishment in soil. Acclimatized plants did not exhibit any morphological abnormalities or variations.

4.3. Essential oils composition

The essential oils isolated from the five *T. caespititius* genotypes (C, CT, SC, G1 and G2) had a strong odour and a yellow color. The shoot cultures provided essential oil yields that were similar to, or greater than, those obtained from field-grown plants. These oil yields ranged from 0.4 to 1.0% (v/fw; $mL.g^{-1}$) in field-grown plants, and 0.2 to 2.1% (v/fw; $mL.g^{-1}$) in *in vitro* shoots.

The essential oil isolated from each sample was a complex mixture in which was identified 63 components, representing ≥ 55% of the total volatiles. The identified oil components are listed in Table 2 in the order of their elution on the DB-1 column, arranged according to the plant genotype with the lowest and the highest percentages found for each component in each oil type. Field material was analyzed only once, while *in vitro* plants were analyzed several times over the course of the experiment (6^{th}, 10^{th}, 12^{th}, 16^{th}, 20^{th} and 24^{th} subcultures after *in vitro* establishment).

The monoterpene fraction was dominant in all samples analyzed, ranging between 41 and 93%, while the sesquiterpene fraction ranged from 2 to 30% (Table 2).

Carvacrol (71%) was the main component of the C field-grown plant essential oils (Table 2, Fig. 3A). The corresponding C shoots essential oils were dominated by carvacrol (20-70%) and carvacryl acetate (6-40%), Fig. 3C.

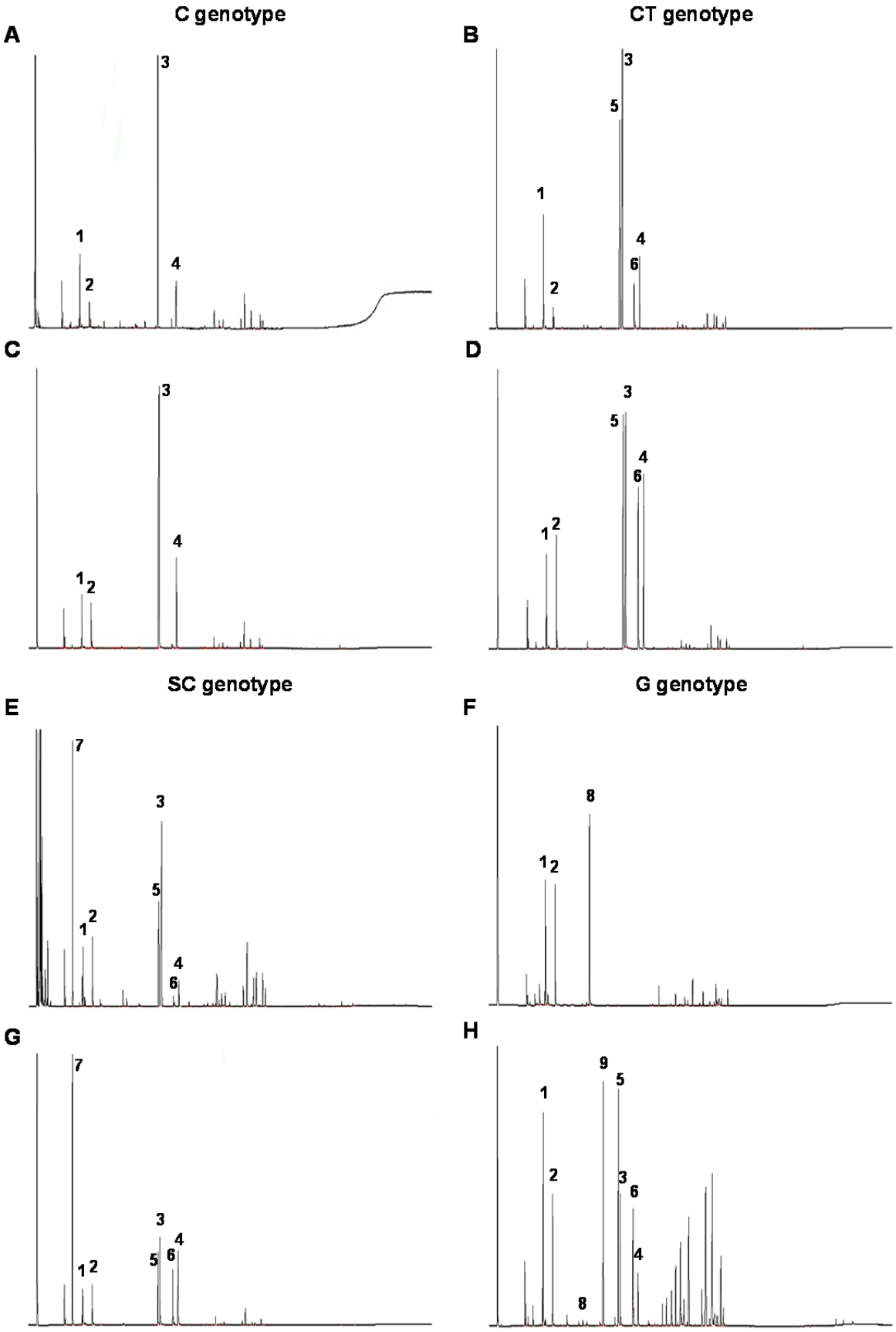

Figure 3. Gas chromatography profiles of the essential oils isolated from four field grown plants (A,B,E,F) and *in vitro* shoots (C,D,G,H) of *T. caespititius*. Main components detected in the essential oils: 1, *p*-cymene; 2, γ-terpinene; 3, carvacol; 4, carvacryl acetate; 5, thymol; 6, thymyl acetate; 7, sabinene; 8, α-terpineol; 9, carvacrol methyl ether.

Table 2. Minimum and maximum percentage range of components identified in the essential oils isolated from five *in vitro T. caespititius* plants cultivated on MS medium supplemented with 0.4 mg.L^{-1} BA and 0.1 mg.L^{-1} IBA (sampled at the 6th, 10th, 12th, 16th, 20th and 24th subcultures after *in vitro* establishment) and from their corresponding field-grown plants. For the G plants, the *in vitro* cultures were established from seeds and higher differences were expected in comparison with the mother-plant.

		C plants			CT plants			SC plants			G plants				
		FG	*in vitro*		FG	*in vitro*		FG	*in vitro*		MP	G1 *in vitro*		G2 *in vitro*	
			Min	Max		Min	Max		Min	Max		Min	Max	Min	Max
Components	**RI**														
Tricyclene	921	t	t	t	t	t	t	t	t	t	t	t	t	t	t
α-Thujene	924	1.7	t	3.6	2.9	0.7	4.1	2.7	1.9	3.8	3.1	0.2	2.2	0.1	3.0
α-Pinene	930	0.6	t	1.1	0.8	0.2	1.0	0.5	0.5	0.9	1.2	0.1	0.8	t	1.1
Thuja-2,4(10)-diene*	940	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Camphene	938	0.1	t	0.1	0.1	t	0.1	0.1	t	0.1	0.4	0.1	0.4	t	0.8
Sabinene	958	0.2	t	0.3	0.1	0.2	0.5	17.9	38.9	48.6	1.2	0.1	0.8	t	0.5
1-Octen-3-ol	961	0.2	t	t	t	t	t	t	t	t	t	t	t	t	0.1
β-Pinene	963	0.4	t	0.3	0.2	0.1	0.4	t	t	t	0.4	t	0.4	t	0.4
n-octanal	973	t	t	t	t	t	t	t	t	t	t	t	t	t	t
β-Myrcene	975	t	t	t	t	t	t	t	t	t	1.5	t	t	t	t
α-Phellandrene	995	0.1	t	0.2	0.1	0.1	0.2	0.2	t	0.1	0.2	t	0.2	t	3.0
δ-3-Carene	1000	0.1	t	0.1	0.1	t	0.1	t	t	0.1	0.1	t	0.1	t	0.1
α-Terpinene	1002	0.4	t	1.0	0.6	0.9	1.8	1.6	0.2	1.2	1.5	0.2	1.4	0.2	2.3
p-Cymene	1003	3.5	0.2	5.5	7.9	3.3	7.5	3.7	3.4	4.9	15.1	2.0	11.3	8.2	20.7
β-Phellandrene	1005	0.2	t	0.2	0.3	0.1	0.4	0.5	t	0.3	t	t	0.3	t	0.3
Limonene	1009	0.2	t	0.2	0.2	0.1	0.3	0.5	t	0.3	2.1	t	0.7	0.3	0.6
γ-Terpinene	1035	1.4	0.1	4.2	1.5	4.1	9.6	4.5	3.3	5.1	14.8	3.9	11.0	6.1	10.2
trans-Sabinene hydrate	1037	0.7	t	1.1	1.0	0.4	1.2	0.4	t	0.6	0.1	t	0.4	t	0.5
2,5-Dimethyl styrene	1059	t	t	t	t	t	t	t	t	t	t	t	t	t	t
p-Mentha-2,4(8)-diene	1064	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t
Terpinolene	1064	t	t	0.1	t	t	0.1	t	t	t	0.4	t	0.1	t	t
cis-Sabinene hydrate	1066	0.1	t	0.1	0.2	t	0.1	0.5	t	0.1	t	t	t	t	t
Linalool	1074	0.2	t	t	t	t	t	t	t	t	t	t	t	t	t
1-Octen-3-ol acetate	1086	0.4	t	0.2	0.1	t	0.1	0.3	t	0.2	t	t	t	t	t
trans-*p*-2-Menthen-1-ol	1095	t	t	t	t	t	t	t	t	t	0.1	t	t	t	t
cis-*p*-2-Menthen-1-ol	1110	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Isoborneol	1132	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Borneol	1134	0.1	t	0.1	t	t	t	t	t	t	0.3	t	t	t	t
Terpinen-4-ol	1148	0.4	t	0.5	0.3	0.2	0.5	1.2	t	1.1	0.6	t	0.3	t	0.5
α-Terpineol	1159	0.1	t	t	0.4	t	0.1	0.7	t	t	28.0	t	0.7	t	0.8
cis-Carveol	1202	0.3	t	0.9	0.1	t	0.2	t	t	t	t	t	t	t	t
Carvone	1206	0.2	t	0.3	0.3	t	0.2	t	t	t	t	t	t	t	t
Thymoquinone	1210	0.2	t	t	t	t	0.2	t	t	t	t	t	t	t	t
Carvacrol methyl ether	1224	0.1	t	0.1	t	t	t	t	t	t	0.1	8.8	13.6	11.3	17.3
2-Phenyl ethyl ether	1228	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Geraniol	1236	0.6	t	0.2	t	t	t	t	t	t	t	t	t	t	t
Bornyl acetate	1265	t	t	t	t	t	t	t	t	t	0.1	0.3	2.4	0.6	2.4
Thymol	1275	0.2	t	1.0	22.9	17.0	25.4	1t	7.7	11.5	t	9.3	17.2	10.8	17.5
Carvacrol	1286	71.1	20.0	69.1	42.4	16.1	27.9	27.7	10.8	18.0	t	4.3	9.9	2.0	10.2
Thymyl acetate	1330				3.4	8.9	15.2	0.6	2.8	6.1	t	3.9	14.0	1.3	7.5

(Cont.)

		C plants			CT plants			SC plants			G plants				
		FG	*in vitro*		FG	*in vitro*		FG	*in vitro*		MP	G1 *in vitro*		G2 *in vitro*	
			Min	Max		Min	Max		Min	Max		Min	Max	Min	Max
Carvacryl acetate	1348	3.6	5.8	39.5	5.8	11.4	22.5	1.7	4.2	8.4	t	1.0	9.0	0.1	2.5
Geranyl acetate	1370	0.1	t	0.9	t	t	t	t	t	t	t	t	t	t	t
β-Elemene	1388	t	t	t	t	t	0.2	0.4	t	0.1	0.4	0.1	0.2	t	0.4
α-Gurjunene	1400	t	t	t	t	t	t	t	t	t	t	t	t	t	t
trans-β-Caryphyllene	1414	t	t	0.1	t	t	t	t	t	t	3.1	0.1	1.3	0.2	1.9
Aromadendrene	1428	t	t	t	t	t	t	t	t	t	t	t	t	t	t
allo-Aromadendrene	1456	0.1	t	t	t	t	t	t	t	t	0.3	t	t	t	t
Germacrene-D	1474	t	t	0.1	t	t	t	t	t	t	1.8	1.3	2.2	0.6	1.9
Eremophillene	1480	t	t	t	t	t	0.1	t	t	t	t	t	t	t	t
Valencene	1484	t	t	t	t	t	t	t	t	t	t	t	t	t	t
trans-β-Dihydroagarofuran	1489	1.5	1.4	5.4	0.7	0.5	1.0	3.0	1.0	2.3	0.3	1.0	2.8	1.2	3.7
α-Muurolene	1494	t	t	t	t	t	t	t	t	t	0.3	t	t	t	t
Hinesene*	1497	t	t	t	0.2	0.1	0.3	0.7	t	0.1	t	t	0.2	t	0.4
γ-Cadinene	1500	0.7	0.3	1.6	0.4	0.2	0.8	1.0	0.1	0.4	1.3	3.0	6.9	0.7	2.0
trans-Calamenene	1505	0.1	t	0.1	0.1	t	t	0.1	t	t	t	t	t	t	t
δ-Cadinene	1505	t	t	0.1	t	t	t	t	t	t	0.1	t	t	t	t
Kessane	1517	0.8	0.6	2.7	0.3	0.2	0.4	0.7	0.4	0.9	0.8	0.5	1.3	0.6	2.1
α-Cadinene	1529	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Elemol	1530	0.1	0.1	0.5	t	t	0.2	0.2	t	0.3	4.1	3.0	6.7	1.9	5.0
UIA Thymus	1597	0.9	0.8	3.9	0.4	0.3	0.6	1.5	0.5	1.1	t	0.6	1.7	0.6	2.2
UIB Thymus	1609	3.2	3.6	16.1	1.4	1.2	2.7	5.8	2.6	5.0	0.5	4.2	9.0	3.0	9.7
τ-Cadinol	1616	1.6	0.8	3.3	1.3	0.6	1.4	2.3	0.4	0.9	3.6	6.5	9.6	1.5	5.1
α-Cadinol	1626	0.1	t	0.2	t	t	t	0.1	t	t	t	0.3	1.2	0.4	1.4
Intermedeol	1626	0.1	t	0.2	1.2	0.5	1.0	2.7	0.4	0.7	t	t	t	t	t
UIC Thymus	1648	1.2	1.6	6.5	0.5	0.5	1.2	2.4	0.7	4.6	0.1	1.3	3.4	1.2	3.7
UID Thymus	1662	0.6	t	0.7	1.0	0.1	0.4	1.2	t	0.3	2.6	0.2	0.8	1.4	5.1
Palmitic acid	1908	t	t	t	t	t	t	t	t	t	t	t	t	t	t
% Identification		92.7	55.4	93.2	95.9	89.9	96.3	86.5	91.2	95.8	87.2	78.7	89.8	66.7	91.5
Grouped Components															
Monoterpene hydrocarbons		9.8	0.3	15.6	16.0	13.0	24.5	33.1	51.8	63.1	41.9	8.0	24.4	14.9	43.3
Oxygen-containing monoterpenes		77.0	40.9	75.3	75.6	65.6	76.4	41.9	28.6	37.9	29.1	35.5	52.2	28.9	47.3
Sesquiterpene hydrocarbons		1.7	0.9	4.6	1.0	0.6	1.7	2.9	0.7	1.4	8.1	6.0	11.2	3.1	7.9
Oxygen-containing sesquiterpenes		3.4	2.4	9.6	3.2	1.7	3.6	8.3	2.0	3.7	8.0	12.9	18.3	5.3	14.3
Fatty acids		t	t	t	t	t	t	t	t	t	t	t	t	t	t
Others		0.8	t	0.2	0.1	t	0.2	0.3	t	0.2	0.1	0.3	2.4	0.6	2.4
Biosynthetic pathways															
Carvacrol/Thymol pathway		80.1	40.3	82.9	83.9	79.8	86.2	48.2	36.6	45.0	30.1	47.5	66.2	43.0	68.9
Sabinene pathway		0.2	t	0.3	0.1	0.2	0.5	17.9	38.9	48.6	1.2	0.1	0.8	t	0.5
α-Terpineol pathway		0.1	t	t	0.4	t	0.1	0.7	t	t	28.0	t	0.7	t	0.8

FG – Field-grown; MP – Mother-plant; RI - Retention Index relative to C_9-C_{20} *n*-alkanes on the DB-1 column; t - trace (<0.05); * - Identification based on mass spectra only; UIA, UIB, UIC, UID Thymus are four unidentified components present in the essential oils.

Carvacrol (42%) and thymol (23%) were the main components of the essential oils of CT field-grown plants (Table 2, Fig. 3B), while shoot cultures were dominated not only by carvacrol (16-28%) and thymol (17-25%), but also by carvacryl acetate (11-23%) and thymyl acetate (9-15%) in similarly large relative amounts (Fig. 3D).

Sabinene (18-49%), carvacrol (11-28%), and thymol (7-12%) were the main components of the essential oils of both field-grown and *in vitro* shoot SC plants (Table 2, Fig. 3E, 3G). However, the percentage of carvacrol (11-18%) was lower in SC shoots essential oils, compared with plants from the field (28%). Sabinene showed an inverse tendency, being higher in *in vitro* shoots (39-49%) than in field-grown plants (18%) (Table 2).

α-Terpineol (28%) was the main compound of the G mother-plant, followed by *p*-cymene and γ-terpinene (15% and 14%, respectively) (Table 2, Fig. 3F). In the seed-derived shoot cultures (G1 and G2) the relative amount of α-terpineol (t-1%) was too low. *p*-Cymene (2-21%) was the main component identified in these oils, followed by thymol (9-18%), carvacrol methyl ether (9-17%), thymyl acetate (4-14%) and γ-terpinene (4-11%) (Table 2, Fig. 3H).

The major components of the essential oils from *in vitro* shoots were fairly stable throughout the *in vitro* period, as shown by periodical analysis (Fig. 4A-E). The slight decrease in carvacrol content occurred in C, CT and SC shoot cultures and was accompanied by an increase in minor monoterpene components. The relative amount of thymol in the *in vitro* cultures was fairly stable and similar to that observed in field plants. The main quantitative differences observed between the oils from C and CT shoots and field-grown plants were related to the compounds with the acetate group. Considering the monoterpenes, no major differences were detected when the compounds were grouped according to their biosynthetic pathway (Table 2). In SC, sabinene biosynthetic pathway was favored, but no big differences were observed in carvacrol/thymol pathway.

The G genotypes showed different profiles of essential oils, in comparison to the mother-plant (which could be due to the genetic variability). In the seed-derived *in vitro* shoots the α-terpineol pathway was less expressed than in the mother-plant, while the opposite was observed in the carvacrol/thymol pathway, where an increase of the relative amounts of *p*-cymene, carvacrol methyl ether, thymol, carvacrol, thymyl acetate and carvacryl acetate (Table 2).

Differences in the volatiles quantity were evident during the rooting process and acclimatization (Table 3). For instance, a considerable increase of *p*-cymene (44%) and α-terpineol (3.1%) were observed in rooted shoots, while in plantlets in pots the increase was lower (22%). In pots, a slight increase of γ-terpinene relative content was also observed (from 10% in culture to 13% in pots). A decrease of carvacrol methyl ether, thymol, carvacrol and thymyl acetate was detected at the rooting stage, as well as in acclimatized plants (Table 3).

Plantlets in pots had less percentage of compounds involved in carvacrol/thymol biosynthetic pathway; however this decrease was not inversely related to the α-terpineol content in the oils (Table 3).

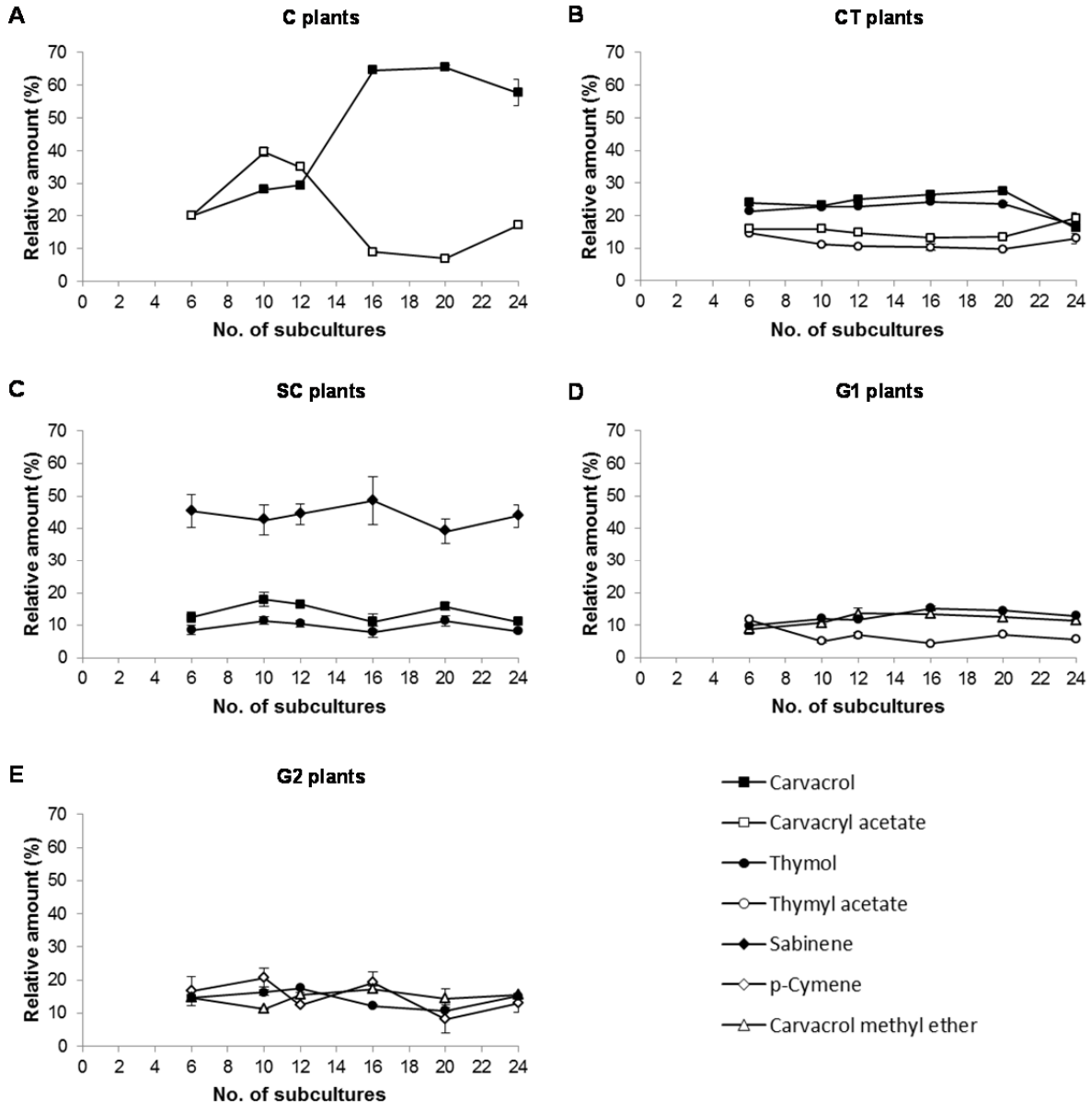

Figure 4. Variation in the relative amount of the main components from *in vitro T. caespititius* essential oils, isolated from shoot cultures on MS medium supplemented with 0.4 mg.L^{-1} BA and 0.1 mg.L^{-1} IBA at 6th, 10th, 12th, 16th, 20th and 24th subcultures after culture establishment (mean ± standard deviation). A) C genotype (carvacrol chemotype). B) CT genotype (carvacrol/thymol chemotype). C) SC genotype (sabinene/carvacrol chemotype). D,E) G1 and G2 genotypes, respectively (mother-plant belonged to α-terpineol chemotype). In some cases SD is too small to be discriminated from the points.

Table 3. Main components (≥ 3%) of the essential oils isolated from proliferating shoots, rooting plantlets and acclimatized plantlets in pots of G2 *Thymus caespititius* genotype.

		Proliferative	Rooting	Acclimatized
Components	**RI**			
p-Cymene	1003	13.0	43.6	21.5
γ-Terpinene	1035	9.7	7.8	13.2
α-Terpineol	1159	0.2	3.1	1.7
Carvacrol methyl ether	1224	15.6	5.5	3.1
Thymol	1275	15.1	5.6	4.2
Carvacrol	1286	5.3	0.2	0.1
Thymyl acetate	1330	3.8	0.1	0.1
Elemol	1530	3.1	1.7	4.5
UIB Thymus	1609	5.7	3.7	7.1
τ-Cadinol	1616	2.2	1.9	4.1
UID Thymus	1662	2.0	2.3	6.6
% Identification		**84.3**	**85.3**	**70.2**
Grouped Components				
Monoterpene hydrocarbons		29.9	60.7	39.8
Oxygen-containing monoterpenes		40.8	14.9	9.3
Sesquiterpene hydrocarbons		4.7	3.7	7.6
Oxygen-containing sesquiterpenes		8.1	5.8	13.0
Fatty acids		t	t	t
Others		0.8	0.2	0.5
Biosynthetic pathways				
Carvacrol/Thymol pathway		62.5	62.8	42.2
α-Terpineol pathway		0.2	3.1	1.7

RI - Retention Index relative to C9-C20 n-alkanes on the DB-1 column; t - trace (<0.05); * - Identification based on mass spectra only; UIB, UID Thymus are two unidentified components present in the essential oils.

5. Discussion

5.1. Shoot cultures

In recent years the increasing commercial importance of secondary metabolites has resulted in a renewed interest in secondary metabolism. In particular, the possibility of using tissue culture technology to alter the production of bioactive plant metabolites appears to be very attractive. Different *in vitro* strategies have been extensively studied with a view to improving the production of plant chemicals (Debnath et al. 2006).

In vitro plant propagation through axillary budding reduces the chances of somoclonal variation as compared with other methods, and is therefore routinely used for the clonal propagation of medicinal plants (Bajaj et al. 1988; Kalemba and Thiem 2004). In the light of this, shoots of *T. caespititius* were used as explants and established in a semi-solid medium to assess the *in vitro* production of essential oils. The high contamination rate observed could be due to the difficulty in surface disinfection

because of the plant architecture (small internodes and a compact structure with sunken trichomes). Nevertheless, to successfully establish and propagate aseptic cultures with a good proliferation rate it was possible.

Shoot number proved to be a reliable method for evaluating the proliferation rate of this species, revealing no major differences between the assessed genotypes, except for G2. On the other hand, fresh weight increase is an indicator of biomass growth and can be used to quantify volatile yield. A survey of the literature on *Thymus* spp. shoot proliferation showed very different results for different species. Coelho et al. (2011) referred to very high proliferation rates for *T. lotocephalus*, attaining over a hundred new shoots per explant. While the studies by Ozudogru et al. (2011) on *T. vulgaris* reported much lower rates, with the mean shoot number varying from 1.6 to 6.0 and 5.1 in *T. longicaulis*. Nordine and collaborators showed that in *T. hyemalis* shoot proliferation was media- and genotype-dependent (0.9- to 6.2-fold), with higher shoot proliferation on MS medium (5.8-fold).

5.2. Essential oil composition

The essential oil composition of *in vitro T. caespititius* shoots was fairly stable among the subcultures and similar to that of the corresponding field-grown plants. The differences observed in the G genotypes, when comparing the mother-plant with the *in vitro* shoots, could either be due to the *in vitro* culture, or to recombination occurring during sexual reproduction, as both cultures were seed-derived. The minor quantitative differences observed in the essential oil composition were probably due to a combination of the *in vitro* conditions and the variability of environmental factors, as previously reported (Casado et al. 2002; Guedes et al. 2003; Figueiredo et al. 2008b; Zuzarte et al. 2010). Preliminary data on *in vitro* propagation of *T. caespititius* adult material reported quantitative differences in essential oil composition as compared with field-grown plants (Mendes et al. 2010). These differences could be due to the fact that the plants tested by Mendes et al. (2010) had been recently established and were not yet stabilized. However, in the present study new plants and an extended subculturing period were used, strengthening the previous suggestions that the differences in the oil composition are only observed shortly after establishment.

The seeds used to establish G1 and G2 *in vitro* cultures resulted from an open cross pollination, so the chemotype and the essential oil composition from the male plant remains unknown. Furthermore, the volatile heritability has not yet been studied for this species. In Mainland Portugal, α-terpineol chemotype is the only chemotype identified, so generally α-terpineol plants only co-exist with others plants of the same chemotype. In the present study these plants were planted in open field (at ESAC) in separate plots, but adjacent to *T. caespititius* plants with different chemotypes, thus allowing open

crossing among genotypes. Some studies revealed that many enzymes involved in biosynthetic steps of the essential oils components are genetically regulated according to the classical Mendelian inheritance (Vernet et al. 1986; Honda et al. 1994). The presence of the dominant monoterpene in *T. vulgaris* is controlled by an epistatic series of five biosynthetic *loci* that has the following sequence: G (geraniol) > A (α-terpineol) > U (thuyanol-4) > L (limonene) > C (carvacrol) > T (thymol) (Vernet et al. 1986). Considering the dominance sequence, a plant with a dominant G allele will have the G phenotype, regardless of whether it has dominant or recessive alleles at the other *loci* (Vernet et al. 1986). If a plant is homozygous recessive to G *loci* (i.e. gg) and has a dominant A allele then it will have the A phenotype, and so on. A plant homozygous for recessive alleles at all five *loci* will have the T phenotype. Probably, monoterpene segregation in *T. caespititius* follows the same pattern. Assuming that α-terpineol chemotype is dominant compared to carvacrol and thymol chemotypes. Considering this hypothesis, the G mother-plant should be heterozygous (Aa) and the selected seeds were recessive (aa) for this characteristic. However, the G1 and G2 *in vitro* shoots did not reveal a specific chemotype, instead showed an increase of the compounds involved in the carvacrol/thymol biosynthetic pathway.

To understand if these differences in oil composition resulted from genetic variation or from *in vitro* stress conditions, rooting and acclimatization were induced in the G2 genotype. During these phases, α-terpineol amount highly increased, although compounds from carvacrol/thymol pathway have also increased. Given this data, it was impossible to determine the plant chemotype. Before drawing any conclusion it is necessary to establish the plants in open field (without controlled conditions) and wait until the flowering period to properly characterize the chemotype. If the profile of the essential oils remains, eventually a new chemotype could be define.

A survey of the literature, looking for essential oil composition comparisons between field-plants and *in vitro* cultured plants, provided variable results. A similar essential oil composition has been reported for other aromatic species of the Lamiaceae family, including *Lavandula viridis* (Nogueira and Romano 2002), *Lavandula pedunculata* (Zuzarte et al. 2010), *Minthostachys mollis* (Chebel et al. 1998), *Mentha spicata* (Hirata et al. 1990), and *Thymus mastichina* (Fraternale et al. 2003a). However, other studies comparing *in vitro* and field-grown plants have reported differences. Arikat et al. (2004) observed slight deviations in major monoterpene proportions between *Salvia fruticosa* microshoots and *in vivo* plants, albeit with the same constituents. In *Agastache rugosa* some differences were observed between the essential oil composition of *in vitro versus* field-grown plants, with an almost complete absence of estragole in *in vitro* shoots (Zielińska et al. 2011). In *Origanum bastetanum* plantlets the essential oils composition showed some big differences relatively to the mother-plant. *cis*-Tujanol was completely absent in the plantlets and γ-terpinene was the main

component in the oils (Socorro et al. 1998).

Although *in vitro* culture is clearly recognized to be a stressful condition (Casado et al. 2002), shoots grown *in vitro* are continuously exposed to a defined microenvironment, providing nearly optimal conditions for plant proliferation. In contrast, the growth conditions of field-grown plants vary markedly, with lower relative humidity, higher light levels, and septic environments that may account for the synthesis of different amounts of monoterpenes (Juliani et al. 1999). In this study, the decrease in the relative amount of carvacrol in essential oils of *in vitro T. caespititius* shoots was followed by an increase in a few monoterpenes, such as γ-terpinene and p-cymene. These components are biosynthetically related to carvacrol, whose synthesis derives from γ-terpinene and *p*-cymene, as described by Poulose and Croteau (1978) for *T. vulgaris*. A similar biosynthetic pathway has been proposed for *T. vulgaris* by other authors (Dewick 2002; Crocoll 2011), as it has for *Origanum vulgare* (Johnson et al. 2004; Crocoll et al. 2010, Crocoll, 2011). In *O. vulgare* ssp. *hirtum*, environmental and ontogenetic variations affected the relative content of these metabolites, with carvacrol becoming dominant over *p*-cymene during plant development (Johnson et al. 2004). In the latter study, γ-terpinene was also found to decrease from younger to older leaves. The increase observed in the precursors of carvacrol in *T. caespititius* essential oils of *in vitro* shoots, compared to field-grown plants, could therefore be the result of some rejuvenation effect associated with the *in vitro* culture and high cytokinin/auxin ratio. The increase in carvacryl acetate and thymyl acetate in *in vitro* culture could be the result of a stress response to *in vitro* conditions, as these compounds result from post-enzymatic modifications (acetylation) of the primary structure that leads to carvacrol and thymol (Keszei et al. 2008).

In SC *in vitro* shoots, the increase in sabinene and decrease in carvacrol compared with field-plants suggest that in this genotype the sabinene biosynthetic pathway may have been affected by environmental conditions. Since sabinene and carvacrol arise independently via an alternative form of cyclization of the common precursor geranyl diphosphate (Shütte 1998), the observed changes in their relative proportions could be explained by differential modifications in the levels or activities of various cyclase enzymes in the cultured shoots (Sudriá et al. 1999). This could happen indirectly through differential regulation of the given metabolic step, or directly through the loss, or function alteration, of an enzyme (Keszei et al. 2008). In *O. vulgare* growing under different field conditions, the sabinene and carvacrol contents were quite stable, thus suggesting that these biosynthetic pathways may be under strong genetic control in this plant (Tibaldi et al. 2011).

6. Conclusion

The use of *in vitro* shoot cultures to study the production of essential oils is only starting. Controlled growth conditions reduce the sources of variability known to affect essential oil composition, thus making *in vitro* shoot cultures a successful system.

In conclusion, the work here reported showed that *in vitro* proliferating *T. caespititius* shoots initiated from axillary buds produce the same secondary metabolites found in field-grown plants, albeit with minor quantitative differences. In addition, the large biomass increase obtained *in vitro* opens up the possibility of using a large-scale *in vitro* system for metabolite production.

The conditions established in this way also open up the possibility of using *T. caespititius* shoot cultures as an experimental model to investigate the genes involved and the regulation mechanisms affecting the metabolism of terpene compounds.

7. References

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Chapter III

Monoterpene biosynthesis in *Thymus caespititius*: cDNA isolation and functional analysis of three monoterpene synthases

Part of the results presented in this chapter were previously published.

Mendes MD, Barroso JG, Oliveira MM, Trindade H. **2014**. Identification and characterization of a second isogene encoding γ-terpinene synthase in *Thymus caespititius*. DOI 10.1016/j.jplph.2014.04.001.

1. Abstract

Terpenes are one of the largest and most diversified families of natural compounds. These compounds have found numerous industrial applications (in pharmaceutical and food industries), the molecular basis of their synthesis in plants has, until now, not been fully understood. Plant genomes have been shown to contain dozens of terpene synthase genes. The search for terpene synthase genes was performed in five *in vitro Thymus caespititius* genotypes. Three *Tctps* genes were found in these plants, two genes (*Tctps2* and *Tctps4*) coding for γ-terpinene synthase and one coding for α-terpineol synthase (*Tctps5*). The two isogenes and the *Tctps5* were isolated and functionally characterized in different plant genotypes. Alignment of the three *Tctps* revealed a transit peptide much shorter in *Tctps4* than in *Tctps2* and *Tctps5* (3-4 aa instead of 46-47 aa). Also, the *Tctps4* (1665 bp) open reading frame is shorter than *Tctps2* (1794 bp) and *Tctps5* (1806 bp). The amino acid sequence of the three monoterpene synthases shared 77% pairwise identity. The fact that *T. caespititius* carries two isogenes for γ-terpinene synthase, suggests gene duplication along the evolutionary process, followed by mutations leading to the differentiation of both genes. These mutations didn't compromise protein activity. Site-directed mutagenesis in Tctps2 revealed that Arg-505 residue is important for protein stability, and the change to a nonpolar hydrophobic amino acid (Gly) disrupted that stability. A high accumulation of transcripts from the *Tctps* genes was found in shoots of *in vitro* plantlets, while in roots they could not be detected. Still, γ-terpinene levels in aerial parts were reduced, probably due to fast conversion into carvacrol and thymol, the main components of *T. caespititius* essential oils. This study is a contribution to the identification of terpene synthase genes in Lamiaceae.

2. Introduction

As mentioned in Chapter I, essential oils are a complex mixture of terpenoids (mono-, sesqui- and diterpenes) and phenylpropanoids, among others. The volatility and quantity of these compounds contribute to the particular properties of many spices and herbs (Gang et al. 2001; Figueiredo et al. 2008). The different chemical composition of the essential oils is determined by physiological conditions of the plant organ, the time of day and environmental conditions, such as temperature, light intensity, among others (Figueiredo et al. 2008).

Terpenoids are the largest, most diverse class of plant natural products (approximately 65,000 have been identified) and they play numerous functional roles in primary metabolism and in ecological interactions (Trapp and Croteau 2001; Kampranis et al. 2007, Degenhardt et al. 2009; Koksal et al.

2011; Oldfield and Lin 2012). The initial substrates for the biosynthesis of the terpenes are the simple C5 precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which in higher plants is produced through two independent pathways located in separate intracellular compartments. The mevalonic acid (MVA) pathway locates in the cytosol and the methylerythritol phosphate (MEP) pathway in the plastids. The activity of three prenyltransferases produces the direct precursors of terpenes, the linear prenyl diphosphates geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20). Terpene synthases (TPS) are the primary enzymes responsible for catalyzing the formation of hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15) or diterpenes (C20) from the substrates DMAPP, GPP, FPP and GGPP, respectively (Trapp and Croteau 2001; Tholl 2006; Cheng et al. 2007).

Many TPSs have been characterized to date, some catalyzing the formation of a single terpene compound, although many TPS enzymes have the ability to synthesize complex product mixture with high regio- and stereospecific (Davis et al. 2000; Tholl 2006; Degenhardt et al. 2009). The tremendous range of possible variations in the carbocationic reactions (cyclizations, hydride shifts, rearrangements, and termination steps) catalyzed by the TPSs explains the wide range of possible products (Trapp and Croteau 2001; Roeder et al. 2007; Fischer et al. 2013a). Furthermore, enzymes such as cytochrome P450 monooxygenases and oxidoreductases are also involved in further modifications of the terpene skeletons, yielding the extremely high diversity of terpene compounds found in nature (Daviet and Schalk 2010; Crocoll 2011). Plant monoterpene synthases [600-650 amino acids (aa)] are longer than sesquiterpene synthases (550-580 aa) due to an N-terminal signal peptide that targets the initial translation product towards the plastids (Turner et al. 1999). The metal ion binding motif, DDxxD is an aspartate-rich region found in all isolated plant terpene synthases and it's the best known structural motif of the terpene synthase (Degenhardt et al. 2009).

Until now, research on TPS has focused on a few species with economic importance, including several species from Lamiaceae, namely *Lavandula* spp. (Landmann et al. 2007; Muñoz-Bertomeu et al. 2008; Lane et al. 2010; Demissie et al. 2011; Demissie et al. 2012; Sarker et al. 2013; Jullien et al. 2014), *Mentha* spp. (Alonso et al. 1992; Colby et al. 1993; Crock et al. 1997; Turner et al. 1999; Crowell et al. 2002; Turner and Croteau 2004; Prosser et al. 2006; Wang et al. 2013), *Ocimum* sp. (Iijima et al. 2004a, Iijima et al. 2004b), *Origanum* spp. (Crocoll et al. 2010; Lukas et al. 2010), *Perilla* sp. (Yuba et al. 1996; Ito et al. 2000; Hosoi et al. 2004; Ito and Honda 2007; Masumoto et al. 2010), *Salvia* spp. (Wise et al. 1998; Hölscher et al. 2003; Kampranis et al. 2007; Schmiderer et al. 2010; Yang et al. 2013) and *Thymus vulgaris* (Krause et al. 2013). For *T. vulgaris*, additional information is available in GenBank, (accessions JQ957864; JQ957865; JQ957866, JX997981, JX997982, JX997983, KC461937). In what concerns *Thymus caespititius*, two monoterpene synthases were

recently characterized (Lima et al. 2013), a γ-terpinene synthase and an α-terpineol synthase.

The aim of this study was to characterize *Tctps2* and *Tctps5* genes in the *in vitro* genotypes and to further identify other terpene synthase genes responsible for the chemical polymorphism observed in *T. caespititius* essential oils. The presence of the two γ-terpinene synthase isogenes is described here for the first time, and it may contribute to elucidate the evolutionary process of terpene synthase genes in Lamiaceae.

3. Material and Methods

3.1. Plant experiments and sample collection

For terpene synthase genes isolation, the clonal shoot cultures from five *T. caespititius* genotypes (C, CT, SC, G1 and G2) previously established in Chapter II were used. The shoot cultures were established and maintained *in vitro* for more than one year. These cultures were used to evaluate the expression of *Tctps* genes along the subculture period and in different tissues.

The samples consisting of young leaves were collected from shoots at weekly intervals (1, 8, 15, 22, 30 days). For the roots, samples were collected only at the end of the subculture.

In order to avoid possible circadian influence, sampling was always performed between 2 and 3 h p.m. All samples were frozen in liquid nitrogen immediately after collection and stored at -80ºC until processing.

3.2. DNA and RNA extraction

Genomic DNA was isolated from deep frozen aerial parts (50–100 mg) following the CTAB extraction method described by Doyle and Doyle (1987), modified by Weising et al. (1995) and adapted *to T. caespititius* as previously reported (Trindade et al. 2008).

Plant tissue (approximately 100 mg) was excised from *in vitro* shoot-cultured *T. caespititius* plantlets, ground in liquid nitrogen with mortar and pestle, and the total cellular RNA was extracted using TriReagent® Solution (Ambion®) according to the manufacturer's instructions. Quality and concentration of total RNA were assessed by NanoDrop1000 spectrophotometer (Thermo Scientific) at 260 nm.

For cDNA synthesis, 1 µg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions.

Table 1. Oligonucleotide sequences used for gene amplification, 3'RACE PCR, semi-quantitative PCRs, cloning and mutagenesis.

	Primer ID	Primer sequence (5'-3')
Gene		
γ-Terpinene synthase	TPS2_1Fw	ATGGCTACCCTTAGCATGCAAG
(*Tctps2*)	TPS2_2Fw	GACACTCCTCAGACTGCACGG
	TPS2_3Fw	GATAGAATTGTGGAGTGCTACTTTTG
	TPS2_4Fw	GGCGTGGAAGGAGATGAACACG
	TPS2_1Rv	CGGCGAGGTTTGAATTAGTCC
	TPS2_2Rv	CTCGACAACCGTTCTCTCAGTC
	TPS2_3Rv	TCACACATATGGCTCGAAAATAAGG
γ-Terpinene synthase	TPS4_1Fw	ATGGAAGGTTCAAAACCAGC
(*Tctps4*)	TPS4_2Fw	GATGAAAAAGGAATCGATGATGAG
	TPS4_3Fw	GGCAACGTCGACTGAGAAGGT
	TPS4_1Rv	CCTCTTTGAGTTCGTCTAACATTG
	TPS4_2Rv	ACCTTCTCAGTCGACGTTGCC
	TPS4_3Rv	CCGTGTTCATCTCCTTCCACG
α-Terpineol synthase	TPS5_1Fw	ATGTCTACCATTAGCATACATCATGTGGGC
(*Tctps5*)	TPS5_2Fw	GCAACGCTGCAACTCTACGAAGCATC
	TPS5_3Fw	CGATAGACCAACTTCCATATTACATGCAAC
	TPS5_1Rv	GATGCTTCGTAGAGTTGCAGCGTTGC
	TPS5_2Rv	GTTGCATGTAATATGGAAGTTGGTCTATCG
	TPS5_3Rv	TCATACGTAAGGGTGGAAGAACAAG
3'RACE PCR		
	Oligo d(T)-anchor	GACCACGCGTATCGATGTCGAC$(T)_{16}$VN
	3'-adapter	GACCACGCGTATCGATGTCGAC
Semi-quantitative PCR		
Tctps2	TPS2_1Fw	ATGGCTACCCTTAGCATGCAAG
	TPS2_4Rv	CCAAGAGATGTGAAGTACAAATCCCC
Tctps4	TPS4_1Fw	ATGGAAGGTTCAAAACCAGC
	TPS4_4Rv	CTCATCATCGATTCCTTTTTCATC
Tctps4	TPS5_1Fw	ATGTCTACCATTAGCATACATCATGTGGGC
	TPS5_1Rv	GATGCTTCGTAGAGTTGCAGCGTTGC
Elongation factor 1-α	EF_1Fw	CAACATATCTGAGCCCAAGAGG
	EF_1Rv	GCTCCTTTCCAGACCTCCTATC
Cloning		
Tctps2	IBA_TPS2_Fs_Fw	TGGTAGGTCTCAGCGCATGGCTACCCTTAGCATGCAAGT
	IBA_TPS2_Cs_Fw	ATGGTAGGTCTCAGCGCATGGCCTCACTGCAAGTCGAGG
	IBA_TPS2_Rv	ATGGTAGGTCTCATATCACACATATGGCTCGAAAATAAGGCC
Tctps4	IBA_TPS4_Fs_Fw	ATGGTAGAATTCAGCGAAGGTTCAAAACCAGCGGCGA
	IBA_TPS4_Cs_Fw	ATGGTAGAATTCAGCTCAAAACCAGCGGCGACGAGC
	IBA_TPS4_Rv	ATGGTACCATGGATCATGCATATGTCTCGAACAACAGT CC
Tctps5	IBA_TPS5_Cs_Fw	ATGGTAGGTCTCAGCGCCGTGTCTCCTCGAAAACCGATAC
	IBA_TPS5_Cs1_Fw	ATGGTAGGTCTCAGCGCCCCGCTGAAATAACCCGACGTT
	IBA_TPS5_Cs2_Fw	ATGGTAGGTCTCAGCGC ATAACCCGACGTTCAGGAAACTAC
	IBA_TPS5_Rv	ATGGTAGGTCTCATATCATACGTAAGGGTGGAAGAACAAGT

(Cont.)

	Primer ID	Primer sequence (5'-3')
Site-directed mutagenesis		
	mTPS2_1Fw	GCTGGCTTATGATATTCTCAAAG**G**TCAGGGTTTCAACAGC
	mTPS2_2Fw	CTCAGTTTGAGCTGAAG**G**GAGGGGACGTGCAAAAGG
	mTPS2_3Fw	GCTACATGAAGGACAACAATG**T**CACAGAGAAAGAAGGG
	mTPS2_4Fw	GCTGGCTTATGATATTCTCAAAG**A**TCAGGGTTTCAACAGC
	mTPS2_5Fw	CTCAGTTTGAGCTGAAG**A**GAGGGGACGTGCAAAAGG
	mTPS2_6Fw	GCTACATGAAGGACAACAATG**C**CACAGAGAAAGAAGGG
	mTPS2_1Rv	GCTGTTGAAACCCTGA**C**CTTTGAGAATATCATAAGCCAGC
	mTPS2_2Rv	CCTTTTGCACGTCCCCTC**C**CTTCAGCTCAAACTGAG
	mTPS2_3Rv	CCCTTCTTTCTCTGTG**A**CATTGTTGTCCTTCATGTAGC
	mTPS2_4Rv	GCTGTTGAAACCCTGA**T**CTTTGAGAATATCATAAGCCAGC
	mTPS2_5Rv	CCTTTTGCACGTCCCCTC**T**CTTCAGCTCAAACTGAG
	mTPS2_6Rv	CCCTTCTTTCTCTGTG**G**CATTGTTGTCCTTCATGTAGC

3.3. TPS gene isolation and RACE

For *Tctps2* and *Tctps5* genes amplification, primers were designed based on the open reading frame (ORF) of previously described *Tctps2* and *Tctps5* from *T. caespititius* (Lima et al. 2013) and used for amplification of this gene from *in vitro* shoots (Table 1).

For isolation and identification of *Tctps* genes, the initial PCR primers were designed from the alignment of several terpene synthase genes identified in Lamiaceae species (GenBank accession number AF051900, AF051901, DQ785794, EF495245), using Geneious software v5.3 (Drummond et al. 2010). Later, it was observed that the sequences obtained were similar to two sequences identified in *Origanum vulgare* (GU385971 and GU385972, Crocoll et al. 2010) and these two were also used for primer design. Specific primers were designed for amplification of this putative gene, later entitled *Tctps4* (Table 1).

The 3'-end of *Tctps* genes was isolated with the 3' Rapid Amplification of cDNA End (RACE) technique. 3' RACE takes advantage of the natural poly(A) tail found in mRNA as a generic priming site for PCR. In this procedure, mRNAs were converted into cDNA using reverse transcriptase and an oligo-dT adapter primer (Table 1). Specific cDNA was then amplified by PCR using a gene-specific primer, TPS2_4Fw for *Tctps2* and TPS4_3Fw for *Tctps4,* that anneals to a region of known exon sequences and an adapter primer that targets the poly(A) tail region, 3'-adapter. This technique allowed the capture of unknown 3´-mRNA sequences that lie between the exon and the poly(A) tail.

PCR amplification reactions were performed on a T-Gradient thermocycler (Biometra) in a final volume of 15 µL, containing 0.75 µL of cDNA or 10 ng gDNA, 1x Green GoTaq Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 mM of each primer and 0.8 U GoTaq DNA polymerase (Promega). The reaction conditions were as follows: 95ºC for 5 min, followed by 35 cycles at 92ºC for 1 min, 60ºC for 45 s and 72ºC for 1 min, followed by a 5 min final extension step at 72ºC.

PCR amplification was confirmed on a 1% agarose gel and later purified with the enzymatic combination of Exonuclease I (ExoI) and Shrimp Alkaline Phosphatase (SAP) (Fermentas) following the manufacturer protocol. PCR products were sequenced at least twice to check primer specificity. Geneious software (Drummond et al. 2010) was used for sequence analyses, edition and basic alignment (BLAST) between the sequences obtained in this study and others available in databases.

In the three genes, polymorphic sites were found, corresponding to ambiguous regions. To isolate this variability, fragments were cloned into the vector pJET using CloneJET PCR cloning kit system (Fermentas) following manufacturer's protocol. PCR amplification (95ºC for 5 min, followed by 35 cycles at 92ºC for 1 min, 60ºC for 45 s and 72ºC for 2 min, followed by a 5 min final extension step at 72ºC) was performed using the primer combination TPS2_1Fw with TPS2_3Rv for *Tctps2* gene, TPS4_1Fw with IBA_TPS4_Rv for *Tctps4* gene, and TPS5_1Fw with TPS5_3Rv for *Tctps5* gene. Amplified sequences with approximately 1.7-1.8 kb were cloned in pJET, and the plasmids were transformed using *E. coli* TOP10 homemade competent cells. After overnight growth at 37ºC on Luria-Bertani (LB) solid medium containing 100 $\mu g.mL^{-1}$ ampicillin, the transformants were analyzed by colony PCR using the vector pJET1.2 primers, forward (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 reverse (5'-AAGAACATCGATTTTCCATGGCAG-3') using the PCR conditions described above. Plasmid DNA of the positive clones was recovered from overnight cultures using GenElute Plasmid MiniPrep kit (Sigma-Aldrich Chemicals), and then sequenced in both strands direction.

Nucleotide sequences from *T. caespititius* genotypes obtained during this study were deposited in GenBank (KF322217 – *Tctps4*_SC (full length sequence) KF322218 – *Tctps4*_C (full length sequence); KF322219 – *Tctps4*_CT (full length sequence); KF322220 – *Tctps4*_G (full length sequence); KF322221 - *Tctps4*_SC.01 (cleaved sequence, active protein); KF322222 - *Tctps4*_SC.02 (cleaved sequence, active protein); KF322223 - *Tctps4*_C.01 (cleaved sequence, active protein); KF322224 - *Tctps4*_C.02 (cleaved sequence, active protein); KF322225 - *Tctps4*_CT.01 (cleaved sequence, active protein); KF322226 - *Tctps4*_CT.02 (cleaved sequence, active protein); KF322227 - *Tctps4*_G.01 (cleaved sequence, active protein)).

3.4. *In vitro* expression and functional analyses of the terpene synthases

The complete open reading frames (ORF's) and the corresponding 5' cleaved sequences for the *Tctps* were amplified by PCR using GoTaq DNA polymerase (Promega) using specific primers sets (Table 1) containing appropriate restriction sites and cloned into the bacterial expression vector pASK-IBA37 plus. Transit peptides were predicted for *Tctps2, Tctps4* and *Tctps5* using the ChloroP prediction site (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson et al. 2000). For *Tctps5* gene,

three cleaved sequences were obtained in order to understand if the size of the pocket influences or not the product specificity. The fragments were run in a 1% agarose gel and recovered with Gel Extraction Kit (Omega). The purified amplicons for *Tctps2* and *Tctps5* genes were digested with *Bsa* I restriction enzyme (NEBiolabs), while for *Tctps4* gene amplicons were digested with *Eco*R I and *Nco* I (NEBiolabs) and ligated into the pASK-IBA37plus expression vector, containing a 6xHis-tag sequence in N-terminal region. The expression constructs were confirmed by sequencing, and the plasmids were transformed into *E. coli* TOP10 homemade competent cells.

Tctps2 and *Tctps4* gene expression was performed for each plant genotype (C, CT, SC, G1 and G2) and *Tctps5* gene expression was only performed for both G genotypes, using two different sequences (clones, designated as .01 and .02). For gene expression, a pre-inoculum was obtained by growing cells overnight in 5 mL LB medium supplemented with 100 $\mu g.mL^{-1}$ ampicillin, at 37ºC and 180 rpm. One and a half mililiters of the culture was added to 100 mL LB medium with the same antibiotic concentration and grown at 37ºC until OD_{600} reached 0.5-0.7. Protein production was induced by adding 2 mM anhydrotetracycline to the cultures, that were then incubated overnight (approximately 16 h) at 18ºC under continuous agitation. The cells were harvested by centrifugation at 4,000*g* for 20 min at 4ºC and the pellet resuspended in 3 mL extraction buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 5 mM $MgCl_2$, 5 mM DTT, 5 mM Na ascorbate, pH 7.0, 0.5 mM PMSF). Bacterial cell wall and membrane were disrupted on ice by sonication for 2 min, power 50% (UP200S, Hielscher Ultrasound Technology). The cell debris was removed by centrifugation at 12,000*g* for 30 min at 4ºC, and the supernatant containing the expressed proteins was collected. Buffer exchange into assay buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 1 mM DTT) was performed using PD-10 Desalting Columns (GE). Enzyme activity was assayed in a 0.2 mL vial containing 13 µL enzyme assay buffer, 4 mM $MgCl_2$ and 70 µL of the soluble protein extract. To prevent GPP dephosphorylation by phosphatases presented in the protein extracts and consequently geraniol formation, phosphatase inhibitors, 0.1 mM Na_2WO_4 and 0.05 mM NaF, were added to the enzymatic assays. The reaction was initiated by adding 20 µM of GPP or FPP (Echelon). Terpene products were collected by solid phase micro extraction (SPME) with a polydimethylsiloxane coated fiber (Supelco). The fiber was exposed for 45 min in the head space above the assay mixture at 4ºC, 21ºC and 42ºC. The adsorbed products were analyzed by GC and GC-MS.

3.5. *In vitro* mutagenesis of terpene synthase open reading frames

Site-directed mutagenesis was performed using the QuickChange method (Stratagene) according to the manufacturer's instructions. The PCR-based mutagenesis protocol was performed with two *Tctps2* cDNA clones (one encoding a functional enzyme and another encoding a nonfunctional

enzyme) into the expression vector pASK-IBA37plus. Three amino acids (D358G, R458G, A474V or G358D, G458R, V474A) were chosen for point mutations in both TPSs clones. Primers containing the desired mutations are listed in Table 1 and the mutated nucleotides are underlined and in bold.

Plasmid DNA of the selected clones was recovered from overnight cultures using Plasmid DNA Purification kit (Macherey-Nagel). PCR amplification reactions were performed in a final volume of 50 µL, containing 25 ng of plasmid DNA, 1x reaction buffer (Stratagene), 1 µL dNTP mix, 0.25 mM of each primer and 2.5 U of *PfuTurbo* DNA polymerase (Stratagene). The reaction conditions were as follows: 95ºC for 30 s, followed by 18 cycles at 95ºC for 30 s, 55ºC for 1 min and 68ºC for 6 min, followed by a 5 min final extension step at 68ºC. One microliter of *Dpn* I restriction enzyme (10 U/µl) was added o the amplification reaction, just prior to incubation at 37°C for 2 h to digest the parental (the nonmutated) supercoiled dsDNA. The *Dpn* I-treated DNAs were transformed into *E. coli* TOP10 homemade competent cells, as mentioned before. The constructs were to confirm successful mutagenesis. The double and triple mutants were also tested and the procedure of heterologous expression in *E. coli* was performed as above described.

3.6. Gas Chromatography (GC)

Terpene synthase assay products were collected by solid phase micro extraction (SPME) using polydimethylsiloxane fiber. Gas chromatographic analyses were performed using a PerkinElmer 8700 gas chromatograph equipped with two flame ionization detectors (FIDs), a data-handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific Inc) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 µm; J&W Scientific Inc.]. Oven temperature was programmed for 45-175°C, at 3°C.min^{-1}, subsequently at 15°C.min^{-1} up to 300°C, and then held isothermal for 10 min; injector and detector temperatures were 240°C and 300°C respectively; and the carrier gas (hydrogen) was adjusted to a linear velocity of 30 cm.s^{-1}. The samples were injected using a splitless technique.

3.7. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS unit consisted of a PerkinElmer Claurus 600 gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific Inc.), and interfaced with a PerkinElmer Claurus 600T mass spectrometer (software version 5.4.2.1617, PerkinElmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm.s^{-1}; splitless;

ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identities of the components were assigned by comparison of their retention indices, relative to *n*-alkane and mass spectra with corresponding data from a home-made library, which was constructed on the basis of analyses of reference oils, laboratory synthesized components and commercially available standards.

For the analysis with the terpene synthase mutated proteins, the assay products were collected by SPME and analyzed by GC–MS (Shimadzu QP2010 Plus, Shimadzu Corporation). The terpenes were separated on an EC5-MS column (30 m x 0.25 mm i.d. diameter, film thickness 0.25 µm; J&W Scientific Inc.). Oven temperature was programmed, 50ºC for 2 min, first ramp 7ºC.min^{-1} to 150ºC, subsequently at 100ºC.min^{-1} up to 300ºC, and then held isothermal for 2 min; splitless injector temperature, 220ºC. The carrier gas was helium, adjusted to a linear velocity of 1 ml.min^{-1}; ionization energy, 70 eV; scan range, 50–350 u; scan time, 1 s. The components identities were assigned with the software GCMS Postrun Analysis (Shimadzu), with the mass spectra libraries Wiley8 (Hewlett & Packard) and Adams (Adams 2007). by using software with Wiley 8 and Shim 2205 MS libraries, as well as by comparison of mass spectra and retention times with those of authentic standards (Sigma-Aldrich Chemicals).

3.8. Semi-quantitative RT-PCR analysis for gene expression profiling

For expression profile analysis of the *Tctps* genes an approximately 500 bp fragment of each of the respective cDNAs was amplified using GoTaq DNA polymerase (Promega) and the primer pair TPS2_1Fw and TPS2_4Rv used for *Tctps2*, TPS4_1Fw and TPS4_4Rv for *Tctps4* and TPS5_1Fw and TPS5_1Rv for *Tctps5*. The *Thymus elongation factor 1-alpha* gene, *ef*-1α, was amplified as a control to estimate if equal amounts of cDNA were used among samples. The primers were designed from the *Origanum*, *OvEF1alpha* (Crocoll et al. 2010). Reactions were performed on a T-Gradient thermocycler (Biometra) with the following cycles: 95ºC for 5 min, followed by 28 or 35 cycles (28 cycles for *Tctps2* and *Tctps5* and 35 cycles for *Tctps4*) at 92ºC for 1 min, 60ºC for 45 s and 72ºC for 1 min, followed by a 5 min final extension step at 72ºC.

Relative abundance of transcripts was determined through quantitative analysis of the digital gel images, using the GeneTools (Syngene, UK) software. The expression reported is the ratio between *Tctps* transcript abundance and *ef-1α* at the same time point.

4. Results

4.1. Identification of *Tctps* genes in *Thymus caespititius* shoot cultures

Total RNA was isolated from five *in vitro* genotypes belonging to distinct chemotypes, carvacrol (C), carvacrol/thymol (CT) and sabinene/carvacrol (SC) and two (G1 and G2) without a specific chemotype. In order to identify terpene synthases responsible for terpene production in *T. caespititius*, RNA was obtained from shoots, since these tissues are rich in glandular trichomes, corresponding to the sites of essential oil biosynthesis and accumulation (Gershenzon et al. 1989; Turner et al. 1999; Schmiderer et al. 2010; Crocoll 2011). RNA was also obtained from roots to compare the expression level of the *Tctps* genes isolated.

For the *Tctps2* and *Tctps5* isolation in these particular genotypes, specific primers (Table 1) were designed based on previous information on these gene from *T. caespititius* published in GenBank (KC181099, KC691294, KC181101, KC181102; Lima et al. 2013). These primers allowed the amplification of the complete open reading frame (ORF) plus the 3'-UTR for *Tctps2*, by RACE-PCR technique. The *Tctps2* ORF was 1794 base pairs long, encoding a protein of 598 aa. The 3'-UTR comprised more than 200 nucleotides. *Tctps5* ORF was 1806 base pairs, encoding a protein with 602 aa. *Tctps5* gene was only isolated and characterized in two *in vitro* genotypes, G1 and G2; in the other three genotypes that had only trace amounts of α-terpineol in its essential oils, it was not possible to amplify this gene.

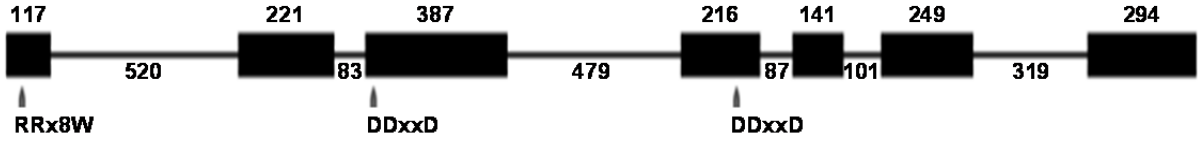

Figure 1. Structure and genomic organization of *Tctps4* genes. Black boxes and lines represent exons and introns, respectively. Box sizes of exons and lines of introns are drawn to scale to their length in nucleotide content as indicated by the arabic numbers. The conserved motifs $RR(x)_8W$ and DDxxD are indicated.

For the *Tctps4* gene, primers were designed based on several sequences from *Salvia* sp. (AF051900, AF051901, DQ785794) and *Rosmarinus officinalis* (EF495245). The first sequences obtained revealed a similarity with the *OvTPS5* from *Origanum vulgare* (GU385971 and GU385972; Crocoll et al. 2010), and these were also considered in primer design. The primers allowed the amplification of an ORF of 1665 nucleotides, with a 3'-UTR larger than 170 nucleotides, encoding a protein of 554 aa.

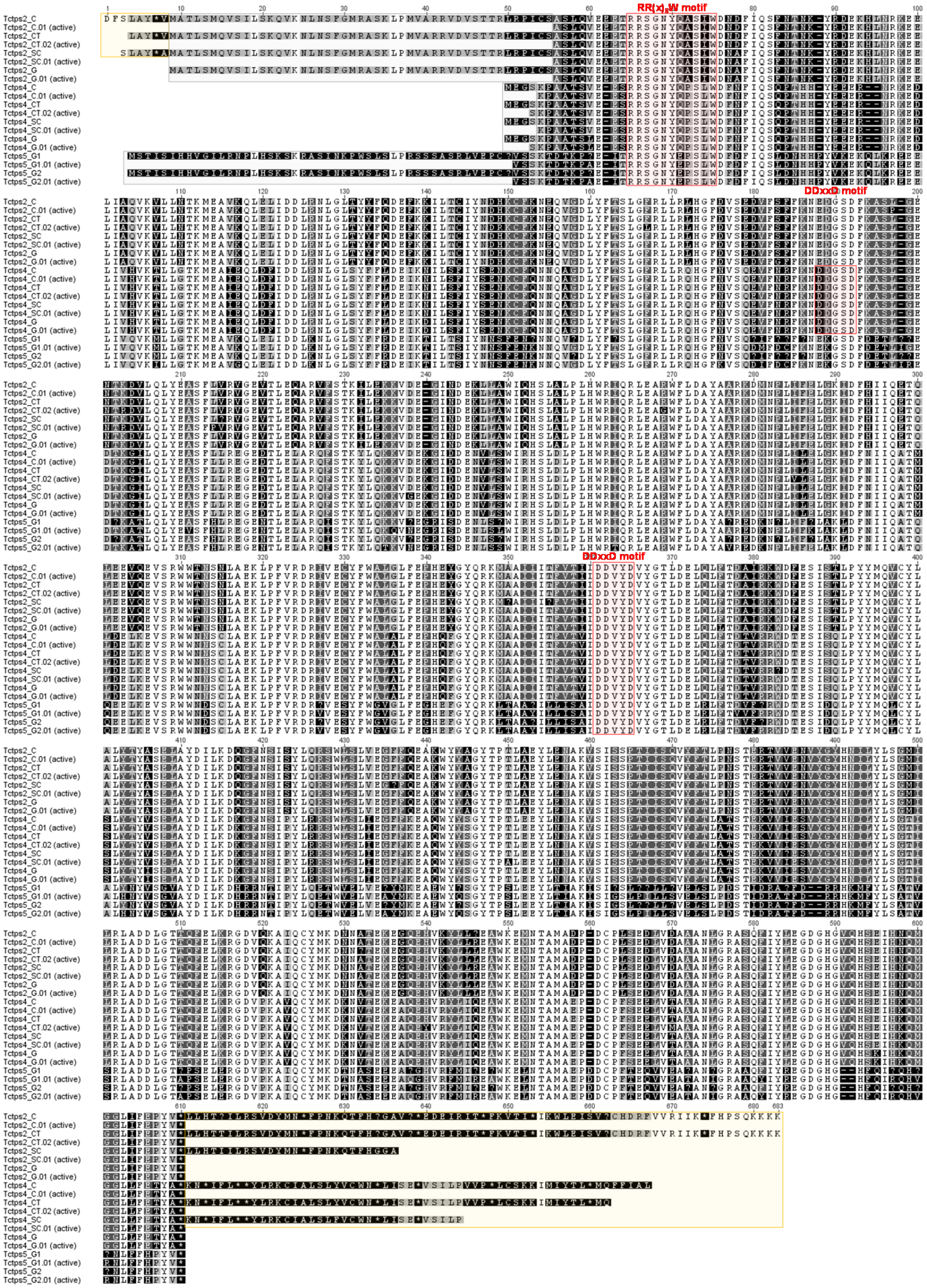

Figure 2. Amino acid alignment of Tctps2, Tctps4 and Tctps5 sequences. Highly conserved residues in the 12 sequences are unshaded; amino acid positions with 80% of similarity are shaded in dark grey; amino acid positions with 60–79 % of similarity are shaded in light grey; and highly divergent amino acid positions are in a black background. Conserved motifs RR(x)$_8$W, and DDxxD, are covered by red boxes. Yellow boxes indicates 5'-UTR and 3'-UTR. Grey boxes delimit predicted transit peptides. Question marks refer to polymorphic sites.

The alignment of *Tctps4* coding regions against gDNA sequences defined exon and intron boundaries, exon and intron sizes and intron placements (Fig. 1). Intron locations were easily identified also by the GC depleted content in these intragenic regions as opposed to the GC enriched transcription regions. Genomic sequences comprised 3254 base pairs organized into seven exons (117, 221, 387, 216, 141, 249, 294 base pairs in length) and six introns (520, 83, 479, 87, 101 and 319 base pairs in length) (Fig. 1). The *Tctps2* and *Tctps5* genomic sequences were previously reported by Lima et al. (2013).

The full-length of three *Tctps* genes was obtained by direct sequencing of the PCR products, revealing the natural polymorphism in these plants. The sequences for *Tctps2* (*Tctps2_C, Tctps2_CT, Tctps2_SC, Tctps2_G2*), were very similar, sharing more than 98% nucleotide identity, the same was observed for *Tctps4* (*Tctps4_C, Tctps4_CT, Tctps4_SC, Tctps4_G*), and for *Tctps5* (*Tctps5_G1, Tctps5_G2*). Due to the intraspecific variability observed in the three genes, complete ORFs were cloned into pJET vector to confirm the nucleotide content. *Tctps2_C.01, Tctps2_C.02, Tctps2_CT.01, Tctps2_CT.02, Tctps2_SC.01, Tctps2_SC.02, Tctps2_G.01, and Tctps2_G.02* revealed more than 99% similarity between the nucleotide sequences and predicted protein. The same result was observed in *Tctps4_C.01, Tctps4_C.02, Tctps4_CT.01, Tctps4_CT.02, Tctps4_SC.01, Tctps4_SC.02, Tctps4_G.01, Tctps4_G.02*. For *Tctps5_G1.01, Tctps5_G1.02, Tctps4_G2.01, Tctps5_G2.02* the similarity was higher than 98%. The three TPS genes shared more than 77% similarity with each other (Fig. 2).

All the TPS genes showed the sequence motifs characteristic of terpene synthases, the double arginine motif, $RR(x)_8W$, which is found in the N-terminal domain of monoterpenes synthases and a highly conserved aspartate-rich DDxxD motif, found in the C-terminal domain. This last motif occurred as DDVYD in all *T. caespititius* genotypes. In *Tctps4* gene, a second DDxxD motif was found between the 135-139 aa residues (Fig. 2).

Transit peptides were predicted for *Tctps2* (47 aa), *Tctps4* (3-4 aa) and *Tctps5* (46 aa) using the ChloroP prediction site (Emanuelsson et al. 2000).

4.2. *In vitro* expression and functional characterization of the monoterpene synthases

Several expression constructs for the three *Tctps* genes were sequenced, revealing high number of isoforms for these genes. Some constructs showed premature stop codons and were not used for expression.

The putative terpene synthases genes were expressed in a bacterial system to evaluate enzymatic activity. *Tctps2* and *Tctps5* full-length clones did not translate into an active protein (data not shown),

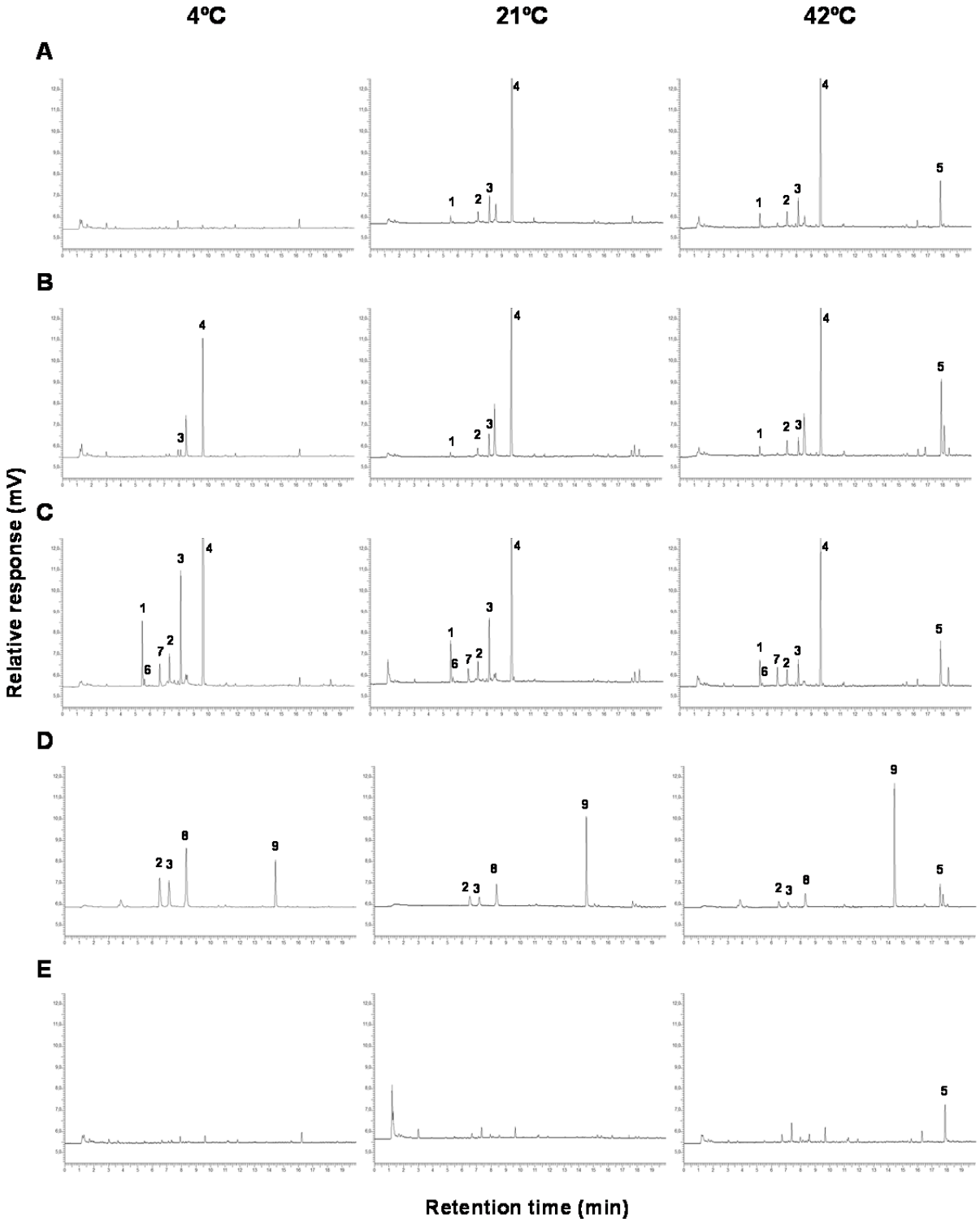

Figure 3. GC chromatograms of the enzymatic products of Tctps2, Tctps4 and Tctps5 expressed in *E. coli* in the presence of GPP at 4ºC, 21ºC and 42ºC. The constructs did not include the transit peptide. A) Products of Tctps2 (Tctps2_C), revealing a very low activity at 4ºC. B) Products of Tctps2 (Tctps2_CT, Tctps2_SC, Tctps2_G), showing product formation at the three temperatures. C) Products of Tctps4. D) Products of Tctps5. E) Example of non-functional terpene synthase. 1, α-thujene; 2, β-myrcene; 3, α-terpinene; 4, γ-terpinene; 5, geraniol; 6, α-pinene; 7, sabinene; 8, limonene; 9, α-terpineol.

but upon deletion of the first 46-47 aa (corresponding to transit peptide) it was possible to recover enzyme activity. The shortened Tctps2 converted the substrate, geranyl diphosphate (GPP), into γ-terpinene and other minor compounds, such α-thujene, β-myrcene and α-terpinene (Fig. 3A, 3B). The GPP feeding following expression of cleaved Tctps5 clones produced α-terpineol and limonene and some minor products, as β-myrcene and α-terpinene (Fig. 3D).

Tctps4 converted GPP into γ-terpinene and the minor products α-thujene, α-pinene, sabinene, β-myrcene, α-terpinene (Fig. 3C). It was active when the original sequence coding for the full-length protein was expressed, as well as with the sequence lacking the predicted plastidial targeting sequence (3-4 aa).

All three *Tctps* genes did not reveal any sesquiterpene synthase activity as shown when the assays were performed with FPP as substrate.

Not all the Tctps isoforms tested revealed a functional protein (Fig. 3E). Only some of all isoforms tested were used in enzymatic assays at different temperatures (4º, 21º and 42ºC). This was performed to get a rough estimate of temperature effect on enzyme activity (Fig. 3). These preliminary results revealed distinctive activities of Tctps2 enzymes assayed at different temperatures. One Tctps2 isoform showed activity only at 21ºC and 42ºC (Fig. 3A), while at 4ºC its activity was reduced. Other Tctps2 isoforms revealed similar activities at different temperatures (Fig. 3B). The same behavior was observed for Tctps4 (Fig. 3C). Tctps5 also showed activity at the three temperatures, although it seemed that product specificity was affected by temperature. At 4ºC the main product detected was limonene and with temperature increase the α-terpineol content also increased. In order to confirm and modify the Tctps5 product duality, the *Tctps5* nucleotide sequence was cleaved once more in the N-terminal extremity, 5 and 8 aa in a total of 49 and 54 aa. These shortened Tctps5, at different temperatures still showed the same product synthesis after GPP feeding; at 4ºC the main product formed was limonene and at 21 and 42ºC the main product detected was α-terpineol (data not shown).

4.3. Similarity and phylogenetic relationships of *Tctps* genes with other Lamiaceae sequences

Comparisons of the putative *T. caespititius* monoterpene synthase genes (*Tctps2, Tctps4* and *Tctps5*) to monoterpene synthase genes from Lamiaceae species (selected 57 genes) showed approximately 55% amino acid sequence identity (Fig. 4). A terpene synthase 3 from *O. vulgare* (ADK73619) was used as outgroup. As expected, Tctps2 and Tctps4 shared the highest amino acid similarity (86%) with others γ-terpinene synthases from *O. vulgare* (ADK73620), *T. caespititius* (AGK88252), *Thymus* x *citriodorus* (AGT29346), *T. serphyllum* (AGT29345) and *T. vulgaris* (AFZ41786, AGS42395) and with a monoterpene synthase from *O. majorana* (AGZ58667) and with

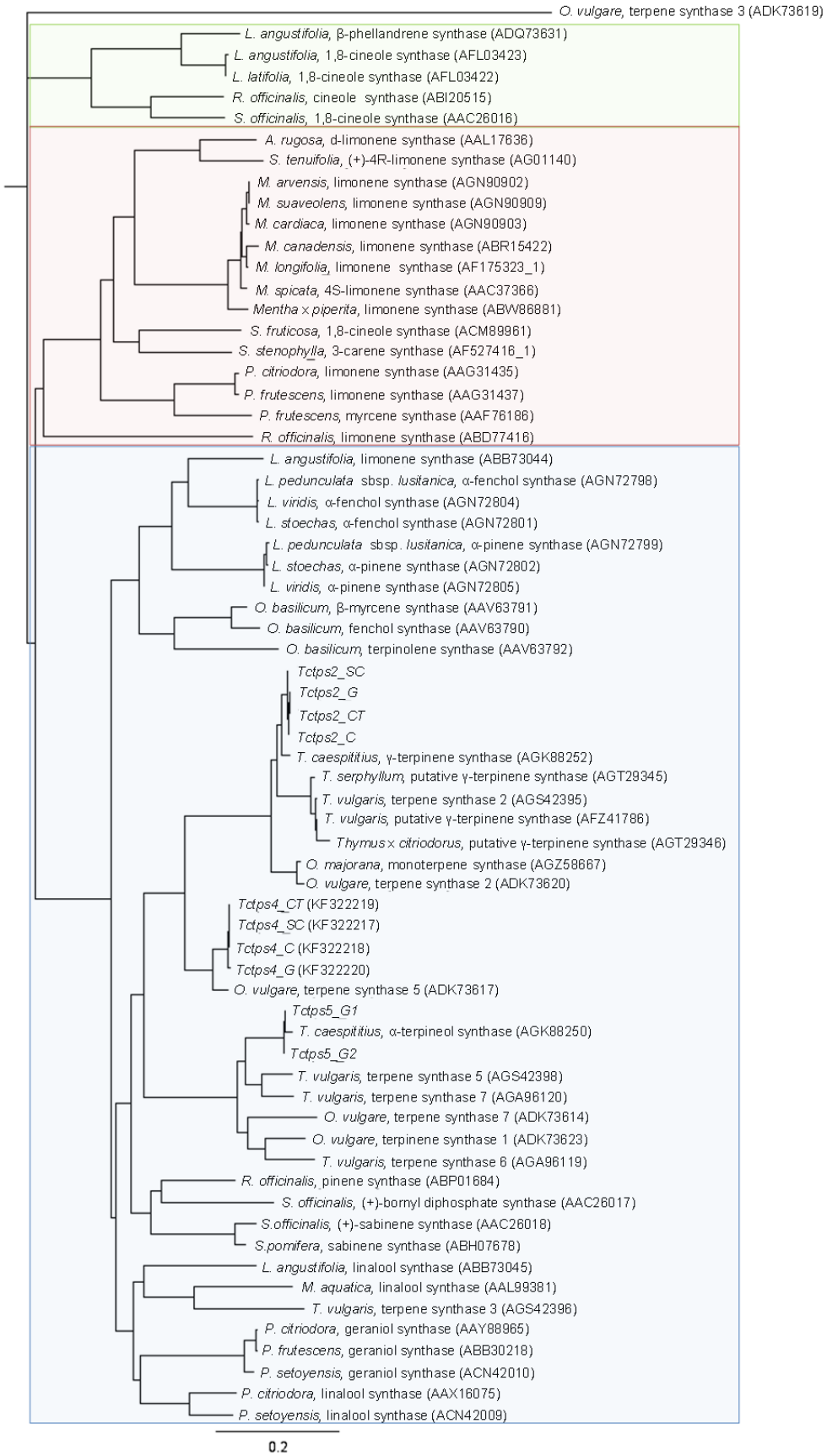

Figure 4. Phylogenetic analysis of Tctps2, Tctps4 and Tctps5 from *T. caespititius* and functionally related terpene synthases from Lamiaceae family. The dendrogram was constructed using the Geneious software v5.3 with the neighbor joining method. A sesquiterpene synthase from *O. vulgare* was used as outgroup. Accessions in parentheses refer to Genbank protein identification.

the terpene synthase 5 from *O. vulgare* (inactive protein, ADK73617). Tctps5 shared 83% of similarity with other α-terpineol synthases from *O. vulgare* (ADK73623), *T. caespititius* (AGK88250) and *T. vulgaris* (AGS42398), sabinene hydrate synthases from *T. vulgaris* (AGA96119, AGA96120) and *trans*-β-ocimene synthase from *O. vulgare* (ADK73614). High identity (69%) was shared with other TPSs, such as (+)-bornyl diphosphate synthase and (+)-sabinene synthase from *Salvia officinalis*, another sabinene synthase from *Salvia pomifera*, and pinene synthase from *Rosmarinus officinalis* (Fig. 4). This analysis confirms that sequence identity among Lamiaceae terpene synthases is relatively high and not necessarily linked to catalytic function, and that despite the sequence diversity that TPS presents, the different sequences share conserved amino acids in both $RR(x)_8W$ and DDxxD domains.

4.4. Site-directed mutagenesis

The 98% amino acid identity between Tctps2_CT.02 (active enzyme) and Tctps2_CT.03 (inactive enzyme) provided an opportunity to identify the residue(s) responsible for the enzyme functionality by sequence comparison and site-directed mutagenesis. Between these two sequences, seven different aa were observed (data not shown), however only three were chosen for mutagenesis (Fig. 5). The selected residues were located downstream of the DDxxD motif.

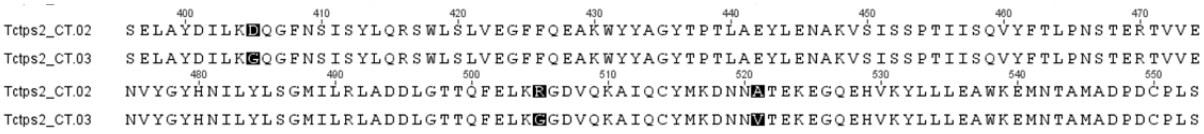

Figure 5. Amino acid alignment of two sequences, Tctps2_CT.02 (active enzyme) and Tctps2_CT.03 (inactive enzyme), suject to site-directed mutagenesis. Highly conserved residues in the two sequences are unshaded; and highly divergent amino acid positions are black.

With recombinant protein expression and enzyme activity analysis, it was found that mutation at these three points affect differently the functionality of the enzyme. The first mutation introduced in Tctps2_CT.02 was Asp-405 to Gly (D405G) to assess whether the presence of a hydrophobic aa in this region contributes to the enzyme activity (Fig. 6). The opposite was conducted in Tctps2_CT.03 (Fig. 7). In both cases the mutation resulted only in geraniol formation. The second mutation was made at the 505 aa. The substitution of an Arg to Gly (R505G) suppressed the synthesis of γ-terpinene (Fig. 6). However γ-terpinene accumulation was observed when in this position a hydrophilic aa like Arg (Fig. 7) was present. The mutation at position 521 [Ala to Val (A521V) or Val to Ala (V521A)] didn´t

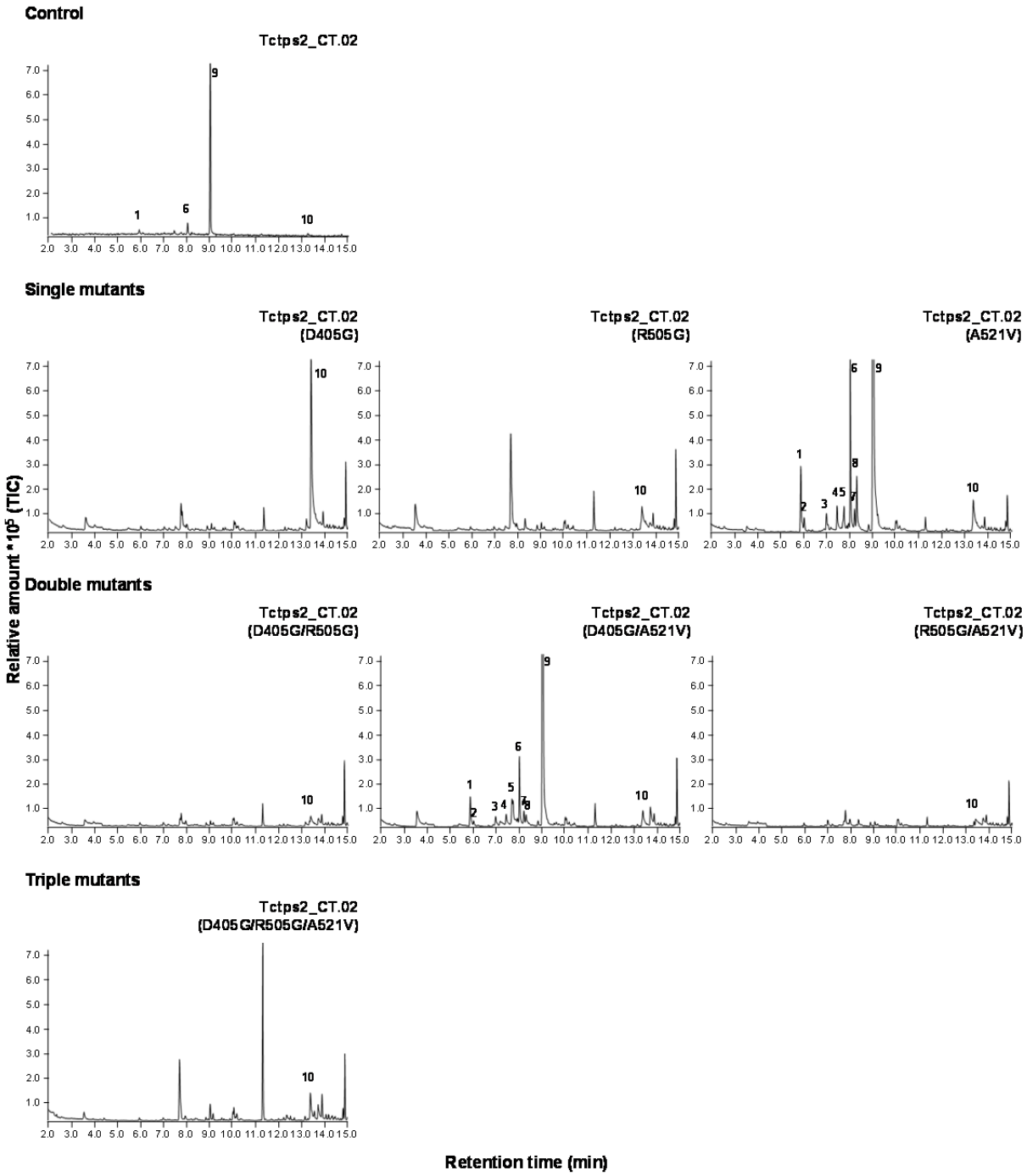

Figure 6. GC-MS chromatograms of products synthesized by Tctps2_CT.02, an active construct (control), and by mutated constructs (single, double and triple mutants) in the presence of GPP. Three amino acids (D358G, R458G, A474V) were chosen for point mutations. The numbers given refer to the compounds identified. 1, α-thujene; 2, α-pinene; 3, sabinene; 4, β-pinene; 5, β-myrcene; 6, α-terpinene; 7, limonene, 8, *p*-cymene; 9, γ-terpinene; 10, geraniol.

affect enzyme activity, the Tctps2_CT.02 remains active, with the synthesis of γ-terpinene and minor products, while the Tctps2_CT.03 was still inactive (Fig. 6 and. 7). The double and triple mutants with a mutation at aa 505 were inactive for Tctps2_CT.02 construct and the opposite was observed for Tctps2_CT.03 construct. The Tctps2_CT.02 double mutant at R405G and A521V resulted in an active

enzyme that produced γ-terpinene and minor products. The data for the first mutation (405 aa) needs confirmation.

The mutation at the position 505 seemed to be important for the enzyme activity (Fig. 6 and 7).

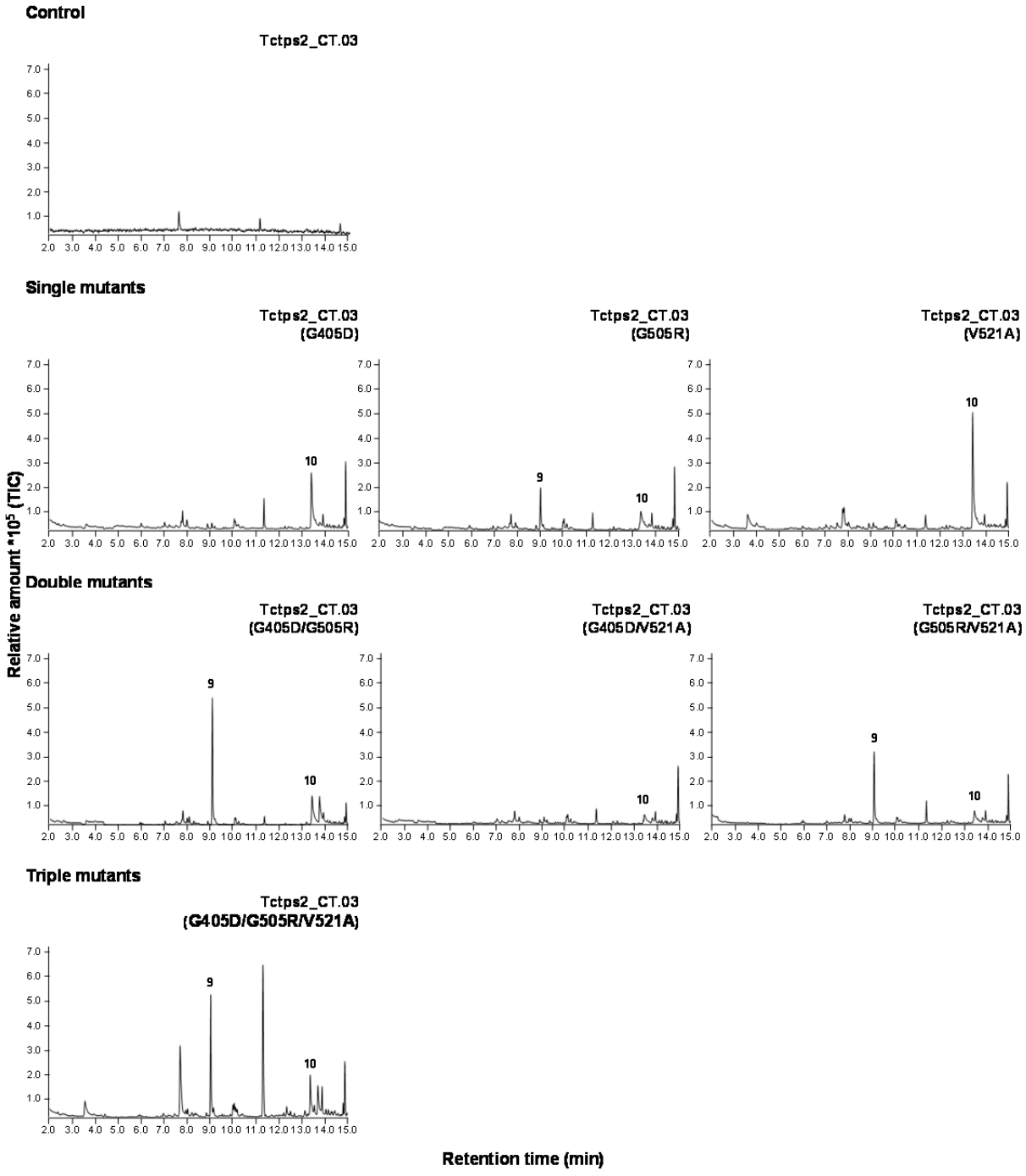

Figure 7. GC-MS chromatograms of products synthesized by Tctps2_CT.03, an inactive construct (control), and by mutated constructs (single, double and triple mutants) in the presence of GPP. Three amino acids (G358D, G458R, V474A) were chosen for point mutations. The numbers given refer to the compounds identified. 9, γ-terpinene; 10, geraniol.

4.5. Transcriptional regulation of the monoterpene synthase genes in *in vitro* cultures

Monoterpene synthase genes expression was determined by semi-quantitative RT-PCR analysis performed using *in vitro* cultures. Total RNAs extracted from *T. caespititius* aerial parts (during the subculture period) and roots were analyzed. As observed in Fig. 8, in roots *Tctps* gene expression was not detected. The optimized semi-quantitative PCRs for the three genes were performed with a different number of cycles, 28 for *Tctps2* and *Tctps5,* and 35 for *Tctps4,* revealing the different expression levels of these genes (Fig. 8). During the subculture period (30 days), the expression pattern of these genes was evaluated in order to understand how terpene synthase genes were expressed during shoot development. *Tctps2* transcripts were always present in the young leaves of the five genotypes shoots; however the *Tctps4* transcripts were always present in C, CT and SC genotypes, while in G1 the expression was only detected at 30 days, and in G2 at 15-22 days no expression was detected or it was too low to be detected by this method. The *Tctps5* transcripts were only detected in G genotypes and were higher at 30 days (Fig. 8). In the first 1-8 days after the subculture, a slight decrease in *Tctps* expression levels was observed in C plantlets, but thereafter the transcript levels increased until the end of the subculture (day 30). For both C and CT shoots, *Tctps2* and *Tctps4* transcripts were higher at 15-22 days. In SC genotype, *Tctps2* and *Tctps4* gene expression levels seemed to be lower than C and CT genotypes and showed a decrease at 15 days (Fig. 8 and 9). In G genotypes the *Tctps* gene expression levels were higher at the end of the subculture, at day 30 (Fig. 9).

5. Discussion

5.1. Identification of *Tctps* genes in *Thymus caespititius* shoot cultures

Shoot cultures are a good method for the multiplication of selected genotypes and chemotypes of several medicinal and aromatic plants (Rout et al. 2000). The *in vitro* cultured plants can be subsequently used for diverse studies, avoiding collection from the natural habitat. Besides their importance in facilitating plant propagation, *in vitro* techniques provide model systems to study the production, accumulation, and metabolism of important metabolites.

T. caespititius produce a large variety of terpene compounds and this diversity could be a result of the expression of multiple terpene synthases and of multiple products formed by certain individual terpene synthases, as reported for others Lamiaceae plants (Iijima et al. 2004a; Crocoll et al. 2010). The existence of chemotypes in this species must result from natural evolution, since this species is not used commercially and thus selective breeding has not been performed. It is likely that most *Thymus* lineages have the genetic potential to synthetize the range of compounds found in the

species, as also suggested by Iijima and collaborators (2004a) for basil plants. However, the molecular regulatory mechanisms by which such diversity is generated (gene silencing, duplications or modifications, differential regulation, post-translational modifications, microRNAs etc.) are still unclear (Iijima et al. 2004a).

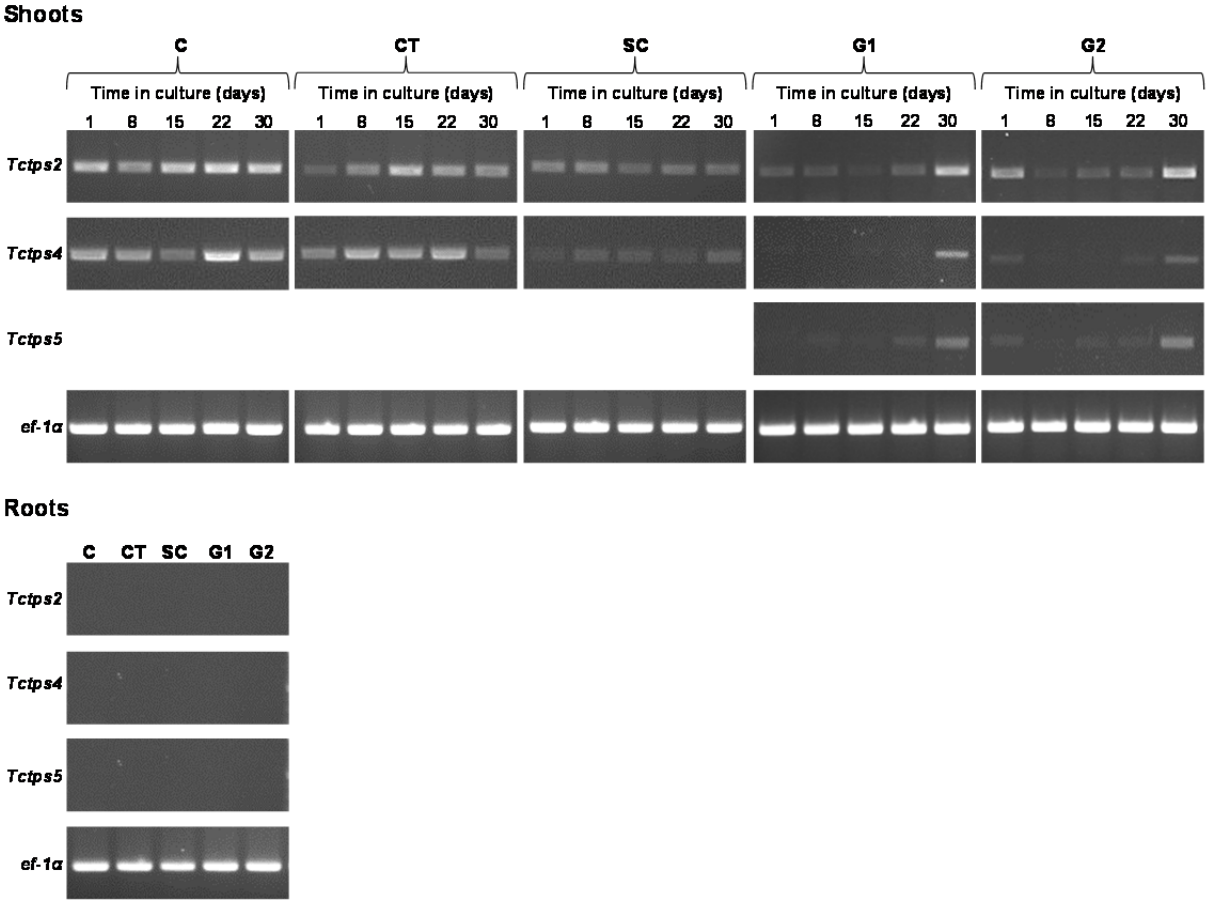

Figure 8. Transcript levels of *Tctps2, Tctps4* and *Tctps5* in shoots of the five *T. caespititius* genotypes during the subculture period (1, 8, 15, 22, 30 days) and in roots. The *Thymus elongation factor 1-α* was used as control. This experiment was repeated twice, using pools of five shoot clusters to minimize individual differences.

To better understand the molecular mechanisms that control chemical differentiation in *T. caespititius,* three monoterpene synthase genes were identified, characterized and their expression analyzed in five *in vitro* genotypes. *Tctps2* and *Tctps5* were previously described in field-grown plants (Lima et al. 2013), so the presence of both genes in the *in vitro* shoots was already expected. *Tctps5* was only detected in G1 and G2 genotypes; which had some α-terpineol in their essential oils while the others only had trace amounts of this monoterpene. The genes isolated from the *in vitro* shoots was not 100% similar to the previous sequence information on *T. caespititius* deposited in GenBank (KC181099, KC691294, KC181101, KC181102) (Lima et al. 2013) revealing some amino acids differences along the sequences. This was already expected considering the plants' natural diversity

and that all the plants used were obtained from natural populations. It is important to point out the fact that the plants used in the present work were different from the ones referred in Lima et al. (2013) and also there is some variability inherent to each plant genotype.

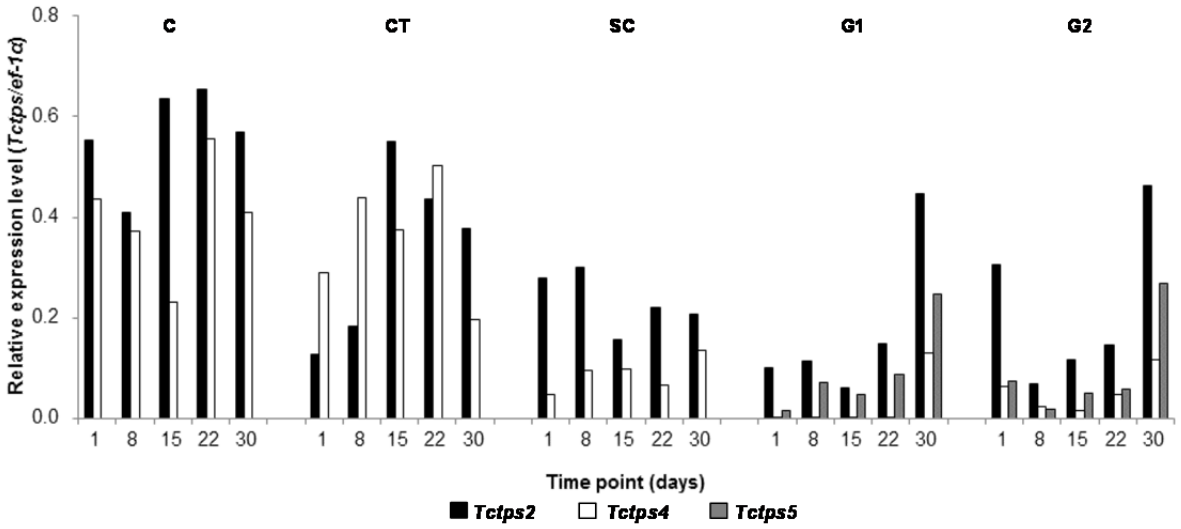

Figure 9. Quantification of RT-PCR products of terpene synthase genes (*Tctps2, Tctps4* and *Tctps5*) in five *T. caespititius in vitro* genotypes during the subculture period (1, 8, 15, 22, 30 days). The bands densities were analyzed with GeneTools software. The *Tctps* relative expression levels were calculated by dividing the band intensity values by the values of *Thymus ef-1α* at the same time point.

In *T. caespititius*, the existence of a sabinene synthase was predictable, because this species has a sabinene chemotype described (Pereira et al. 2000). So, in an attempt to find the genes responsible for this chemotype, some TPS sequences (GenBank accession number AF051900, AF051901, DQ785794, EF495245, GU385971 and GU385972) were used for primer design. The amplified gene, later designated *Tctps4*, was firstly hypothesized to be a sesquiterpene synthase, due to its smaller sequence as compared to monoterpene synthases published in databases. As previously reported, monoterpene synthases are larger than sesquiterpene synthases by 50-70 aa and this difference is due to the N-terminal transit peptide required for plastidial targeting of monoterpene synthases (Bohlmann et al. 1998). In fact, Tctps4 protein was about 40 aa smaller than Tctps2 and Tctps5. However, a high similarity between the three sequences was observed (77%) and an even higher similarity was found between Tctps4 and Tctps2 (88%). *Tctps4* also revealed a high similarity with *OvTPS5* identified from *O. vulgare* (Crocoll et al. 2010) which in heterologous expression, led to the production of an inactive protein.

Genomic information for *Tctps2* and *Tctps5* was previously reported by Lima et al. (2013) and published in GenBank (KC181098, KC691293, KC181096, KC181095). The genomic structure for *Tctps4* comprised seven exons and six introns and can be classified as class III according to a prior

organization proposed for TPS genes (Trapp and Croteau 2001) and into subfamily b according to a more recent classification by Chen et al. (2011). The first, third and sixth introns were larger than those found in *Tctps2* and *Tctps5* and they didn't show any similarity with other sequences published in databases. The introns' phase is defined as the location of the intron before the first, second, or third nucleotide position of the proximate codon and is referred to as phase 0, 1, or 2, respectively (Trapp and Croteau 2001). In the TPS gene family, the introns' phase showed to be conserved. According to Aubourg and collaborators (2002), for all class-III TPS the intron phase was 0 for the first, fifth and sixth introns, 1 for the second and 2 for the third and fourth introns. Structural comparison of the *Tctps4* with *Tctps2* (Lima et al. 2013) revealed conserved exon lengths for the exons 5 and 6; while in the others exons major length differences were detected (3 to 126 nucleotides).

Despite not coding for amino acids, in introns there are exon splicing enhancers that determine mRNA maturation, which is crucial for correct protein translation. Changes affecting these enhancers can be responsible for alternative splicing patterns. Even if the failure to excise an intron does not modify the translation frame, the change in the protein length makes correct folding unlikely, which will end up in a pseudogene with a correct and specific expression, but with the corresponding protein being inactive (Keszei et al. 2008).

5.2. Functional characterization of the monoterpene synthases

As expected the *Tctps2* and *Tctps5* constructs produced an active protein, γ-terpinene synthase and α-terpineol synthase, respectively. Also *Tctps4* expressed clones gave rise to another γ-terpinene synthase. The initial hypothesis that this gene could be a sesquiterpene synthase was not supported, since FPP feeding did not result in volatiles emission indicating only monoterpene synthase activity. So, in *T. caespititius* two distinct γ-terpinene synthase genes and enzymes are present, which are able to convert the substrate GPP into γ-terpinene as the main compound.

The existence of these two isogenes could be a result of gene duplication, in the evolutionary process of the TPS gene family. Gene duplications might be the consequence of short segmental and single gene duplications and of duplications of complete chromosomes or genomes. The most important mechanisms for the origin of duplicated genes are unequal crossing-over and retroposition, (Ober 2010). Unequal crossing-over occurs during prophase I of meiosis, where homologous chromosomes align and exchange chromosomal material of their chromatids by crossing-over. In some cases, this recombination mechanism is disturbed by imprecise pairing of the homologous chromosomes, resulting in nonreciprocal exchange of material and in chromosomes of unequal length. So, a chromosome region is duplicated, forming a tandem repeat on one of the resulting chromatids. (Achaz et al. 2001; Ober 2010). In the case of retroposition, transcribed and spliced mRNA of a certain

gene is incidentally reverse transcribed, and the resulting double stranded cDNA is inserted back into the genome (Wang et al. 2006). Retrogenes are devoid of introns and lack regulatory elements, as the promoters (Zhang et al. 2005). *Tctps2* or *Tctps4* are unlikely to result from retroposition, since in both genes the introns were identified.

The immediate result of gene duplication is the presence of two identical copies. Three different possibilities exist for these gene copies to evolve, 1) both copies remain almost unchanged, 2) both gene copies diverge functionally, and 3) one of the gene copies becomes a pseudogene, because of mutations that silence its function (Graur and Li 1999). Pseudogenisation is assumed to be the most frequent destiny of duplicated genes, since they do not confer a selective advantage and so have a low probability of being fixed in large populations (Zhang 2003). Their average half-life is estimated to be only about 4.0 million years (Lynch and Conery 2003), although, the duplication rates and retention seem to vary depending on the cellular function of the duplicated gene (Roth et al. 2007). Alternatively, both gene copies might retain their original function, forming an invariant repeat. Sequence identity can be conserved in this case through concerted evolution, a mechanism involving gene conversions that results in the homogenization of paralogous sequences (He and Zhang 2005). Both copies are protected against mutations that modify gene function due to their benefit to the organism. Unless the presence of an extra amount of gene product is advantageous, functional divergence between duplicates is usually required for their long-term retention in the genome (He and Zhang 2005). For the development of new enzymatic functions, essentially two different hypotheses have been suggested: the neofunctionalisation and the subfunctionalisation. According to the neofunctionalisation model, one of the copies guarantees the function of the ancestor gene while the second copy is freed from functional constraints and accumulates mutations that might result in a loss of function (nonfunctionalisation) or a gain of function, neofunctionalisation (Ohno 1970). In the subfunctionalisation hypothesis, the ancestral functions of the progenitor gene are partitioned between the duplicates, the joint patterns of activity of the resulting gene copies are equivalent to those of the progenitor gene (Hughes 1994; Force et al. 1999; Lynch and Force 2000; Hughes 2002). In this case, both duplicates remain under permanent selection pressure and accumulate degenerative mutations, causing complementary loss of subfunctions (Roth et al. 2007). Subfunctionalisation can be achieved by modification of the structural gene, although functionality of the gene product will allow only limited modification of the gene structure. More easily, subfunctionalisation can occur by independent modification of the regulatory elements by mutations (Force et al. 1999; Hinman and Davidson 2007) or by epigenetic silencing (Rodin and Riggs 2003). Attending to these possibilities, hypothesizing that the *Tctps2* and *Tctps4* ancestor gene was expressed in different tissues, the expression of the two variants could eventually be restricted to specific tissues or structures. To test this possibility, the

Tctps genes expression should be evaluated in different tissues and in trichomes.

As previously reported, the evolutionary process of TPS gene family involved repeated gene duplication of the ancestral gene followed by divergence by functional and structural specialization (Fryxell 1996; Clegg et al. 1997; Trapp and Croteau 2001). If spontaneous mutation of duplicated genes occurs frequently, the altered gene will be rapidly lost unless it acquires a new and useful function (functional mutation) (Fryxell 1996; Byun-Mckay and Geeta 2007). Crocoll and co-workers (2010) also suggested the existence of gene duplication in *O. vulgare* for the *OvTPS2* and *OvTPS5*, but in this species functional loss of *OvTPS5* could be due to inactivation accompanied by loss of the transit peptide. The existence of TPS isogenes were already reported for *Arabidopsis*, where two root-expressed genes were isolated, encoding 1,8-cineole synthase and (Z)-γ-bisabolene synthase (Chen et al. 2004; Ro et al. 2006).

The transit peptide loss in Tctps4 could result from several mutations or alternative splicing, of the same ancestral gene of Tctps2 (Fig. 4). Tctps4, γ-terpinene synthase, is functional in *in vitro* assays; however it remains to find out if this protein is active inside the plant cell. Since the monoterpene biosynthesis occurs in plastids, the lack of transit peptide in Tctps4 may prevent the traffic of the encoded protein to the plastids, thus preventing its function in the biosynthetic pathway. The lack of transit peptide for monoterpene synthases has already been reported in strawberry, where the *FaNES1* lacked a targeting signal for plastids (Aharoni et al. 2004), indicating that this protein is cytosolic. FaNES1 is responsible for the formation of nerolidol and linalool *in vivo*, which suggests the presence of GPP substrate in the cytosolic compartment (Aharoni et al. 2004; Tholl 2006; Nagegowda 2010). Although all geranyl diphosphate synthases (GDSs) are known to contain a transit peptide for their plastid targeting, there are some ambiguous reports on GDS localization. Evidence for GDS plastidial localization was demonstrated by biochemical studies in *Vitis vinifera* (Soler et al. 1992). However, in *Lithospermum erythrorhizon* cell cultures there was an indication that GDS is cytosolic (Sommer et al. 1995). Bouvier and collaborators proposed that monoterpene metabolism operates in several compartments (plastids, cytosol), within photosynthetic and non-photosynthetic plant cells. However the presence of GDS in the cytosol could not be clearly determined (Bouvier et al. 2000). Contrary to the general belief that all monoterpenes are synthesized in the plastids, some reports indicate that monoterpene synthases can deviate from this pattern and localize in the cytosol as was demonstrated for *FaNES1* and *FvPIN* in strawberry (Aharoni et al. 2004) or can have a dual plastid and mitochondrial localization, as reported for *FaNES2*, also in strawberry (Aharoni et al. 2004) and for α-terpineol synthase in *Magnolia* (Lee and Chappell 2008).

The three monoterpene synthases characterized in this study produced many components identified in *T. caespititius* essential oils, such as α-thujene, α-pinene, sabinene, β-myrcene,

α-terpinene, γ-terpinene and geraniol. The essential oil main components, carvacrol and thymol were not directly produced by these enzymes, but are predicted to be synthesized from γ-terpinene (the main product of *Tctps2* and *Tctps4* genes) via *p*-cymene (Paulose and Croteau 1978; Dewick 2002; Johnson et al. 2004; Crocoll et al. 2010; Crocoll 2011). γ-Terpinene is most likely converted to carvacrol and thymol by one or more cytochrome P450 oxidases, catalyzing a hydroxylation similar to that described for (-)-S-limonene in menthol biosynthesis in *Mentha* sp. (Lupien et al. 1999; Haudenschild et al. 2000). Cytochrome P450 monooxygenases (CYP450) are important in the diversification of terpenoids through oxidative functionalization. These proteins are a very large class of enzymes that utilize NADPH or NADH to cleave atmospheric oxygen reductively while oxidatively functionalizing the substrate (Schuler and Werck-Reichhart 2003, Keeling and Bohlmann 2006).

The sabinene produced by both Tctps2 and Tctps4 enzymes was not sufficient to justify this monoterpene level found in the sabinene chemotype (SC genotype). Despite the reports of a sabinene synthase activity in *O. vulgare* (Crocoll et al. 2010) and *Salvia* sp. (Wise et al. 1998; Kampranis et al. 2007), no sabinene synthase gene could be identified in the present study in *T. caespititius*.

Enzymatic assays using protein extracts were performed at three temperatures, 4ºC, 21ºC and 42ºC and in these it was observed that some Tctps2 isoforms were more susceptible to temperature variation than Tctps4. Tctps5 seemed to present product duality at different temperatures, yielding mainly limonene at low temperatures and α-terpineol at 21ºC and 42ºC. The production of significant amounts of limonene is the result of the failure to hydroxylate the α-terpinyl cation, which is instead converted to limonene by proton elimination (Kampranis et al. 2007). This duality could be due to the protein folding at lower temperatures, reducing water penetration in the active pocket, thus leading to lower production α-terpineol (personal communication of Professor Jörg Degenhardt). The three shortened Tctps5 were insufficient to clarify if the pocket size or conformation was altered. However, considering that no differences in product formation were detected one can assume that no major changes happened. To understand this question, more studies are required, namely kinetic assays with isolated protein at different temperatures and product quantification. It was interesting to find enzyme activity at 4ºC, probably indicating that essential oil production and accumulation occurs in nature at a very wide temperature range. Further *in vivo* studies are also needed to confirm the functionality of the whole biosynthetic pathway at varying temperatures.

Tctps4 had a second DDxxD motif in the N-terminal domain, probably resulting from spontaneous mutations since only one nucleotide was different, as compared to Tctps2. This extra domain might be functional and able to bind divalent ions, which could turn the enzyme more active and more efficient. Further studies are necessary to evaluate if this extra domain is active or not, and to assess how it may affect the product synthesis. In *Abies grandis*, a γ-humulene synthase with two DDxxD motifs

located on opposite sides of the active site was found, suggesting that substrate binding in two different conformations results in different sets of products (Steele et al. 1998).

As mentioned above, several *Tctps* were obtained and some showed premature stop codons while others showed to encode a nonfunctional protein. Studies of the maize TPS4 and TPS5 enzymes also demonstrated that alleles encoding nonfunctional terpene synthases were transcribed (Köllner et al. 2004). A similar situation was found by analyzing the natural variation of floral terpene volatile profiles in different *Arabidopsis* ecotypes. Here, the loss of sesquiterpene volatile emissions from flowers of particular ecotypes was attributed to mutations or to posttranscriptional or translational regulation of two florally expressed TPS alleles rather than to differences in their transcription profiles (Tholl et al. 2005; Tholl 2006).

5.3. Similarity and phylogenetic relationships of *Tctps* genes with other Lamiaceae sequences

The three monoterpene synthases of *T. caespititius* grouped together in the same sub-cluster with other proteins of the same species and of *Origanum* spp., and *Thymus* spp., showing the very close relationship between the two genera. It is known that terpene synthases of the same species are generally more related to each other than to other enzymes from different species but with the same activity (Tholl 2006). Within a species, TPS diversity can be related to adaptive evolutionary process of an ancestral gene copy divergence, in structure and in function, thus leading to a high TPS diversity (Trapp and Croteau 2001).

5.4. Site-directed mutagenesis

Models for related TPS proteins have been tested using domain swapping and site-directed mutagenesis to identify regions and amino acid residues in the region that control product specificity (Tholl 2006). Site-directed mutagenesis and domain swapping can introduce subtle changes in the amino acid residues at the enzyme active site, which can influence substrate binding conformation and the participation of side chain functional groups in catalysis (El Tamer 2002). Despite the overall high structural similarity of TPS enzymes, the identification of individual amino acids that are correlated with specific mechanistic steps has been achieved to date in only a few cases, and might remain a difficult task given the TPS proteins diversity (Tholl 2006).

The high sequence similarity between Tctps2_CT.02 (active enzyme) and Tctps2_CT.03 (inactive enzyme) facilitated the use of site-directed mutagenesis to ascertain which amino acid residues are responsible for the enzyme activity. Site-directed mutagenesis was thus used to exchange three different residues present downstream of the DDxxD motif in two *Tctps2* gene, one leading to an

active protein and the other to an inactive one. The enzyme activity was then monitored in an *E. coli* expression system. Alteration of the amino acid residues 521 (A521V or V521A) alone or together with 405 (D405G or G405D), did not affect the product profile, but the change of Arg 505 to Gly (R505G) led to an inactive protein, while the opposite (G505R) restored the enzyme activity. Mutagenic studies demonstrated that a single amino acid substitution (R505G) in Tctps2 is sufficient for protein stability or correct folding of the active site. The alignment between the Lamiaceae monoterpene synthases and the *Tctps* genes isolated in this study (Fig. 4) revealed that the Arg residue is conserved in almost all sequences. Exceptions were the terpene synthase 6 from *T. vulgaris* (AGA96119) with a Thr instead, and the terpene synthase 3 and 7 from *O. vulgare* (ADK73619, ADK73614) where Gln or Lys respectively, were found in this position. Considering that, the amino acids Arg, Thr, Gln and Lys are polar and hydrophilic, the alteration of Arg to any of the other three above- mentioned aa should not affect the protein stability. But, whenever the change is for a nonpolar and hydrophobic amino acid, such as Gly, alterations are expectable at the protein active site. This points for a general role of this amino acid position in the stereocontrol of terpene synthases.

In terpene synthases, acidic (Arg and Gln) or aromatic amino acids are known to stabilize positive charges and carbocationic intermediates, whenever positioned around the active site. In the case of acidic amino acids, this is due to the presence of a carboxylate anion. In the case of aromatic amino acids, the π-electrons of the aromatic ring and the phenolic oxygen can readily accept a proton. Thus, they stabilize a cation and participate in the rearrangements of terpene structures (Starks et al. 1997; Bohlmann et al. 1998; Seemann et al. 2002; Maruyama et al. 2002). The amino acid residues in the enzyme that stabilize the specific position of the positive charge in the carbocationic intermediates are likely to be highly important for product outcome (El Tamer 2002).

The effect of the mutation at position 405 of Tctps2_CT.02 needs confirmation, since in this study it encodes a nonfunctional protein; however the double mutant A521V/D405G translates into an active protein.

To identify which regions of the protein control product specificity, domain swapping experiments were performed to demonstrate that fragments of several hundred amino acids can determine catalytic activity (Peters and Croteau 2003; El Tamer et al. 2003). Mutagenesis of single amino acids in terpene synthases showed that alterations in the DDxxD motif, in the double Arg motif found in the protein N-terminus, and in active site tyrosine residue dramatically altered both the kinetics and the product specificity of the enzymes (Rising et al. 2000; Little and Croteau 2002; Degenhardt et al. 2009).

Several approaches were therefore undertaken to identify the amino acids that have important impact on the catalytic reaction mechanism, and affect the product formation. Domain swapping experiments of terpene synthases from *Salvia officinalis* and *Citrus limon* indicated that C-terminal

region influences the product specificity (Back and Chapell 1996; El Tamer et al. 2003). Site-directed mutagenesis approaches allowed the identification of key amino acids of the active site, as well as distant from it, which altered the product spectrum of several terpene synthases, such as pinene synthase and γ-humulene synthase from *Abies grandis* (Hyatt and Croteau 2005; Yoshikuni et al. 2006) and 5-epi-aristolochene synthase from *Nicotiana tabacum* (Greenhagen et al. 2006). Kampranis and collaborators (2007) tried to elucidate what are the main structural elements contributing to substrate and product specificity in a synthase. To do that they studied similar enzymes in the related species *S. fruticosa* (1,8-cineole synthase), *S. officinalis* (bornyl pyrophosphate synthase, 1,8-cineole synthase, sabinene synthase) and *S. pomifera* (sabinene synthase). Amino acid sequences in two regions were different in *S. fruticosa* and *S. pomifera*, which attracted attention and were then mutagenized. Sf-CinS1 was successfully converted to a sabinene synthase with a minimum number of substitutions (Kampranis et al. 2007). While in *S. pomifera* sabinene synthase, two mutations altered the 1,8-cineole and α-terpineol production, resulting in a three-fold higher production of α-terpineol over 1,8-cineole. An additional mutation shifted the enzyme to the production of α-terpineol and prevented the cyclization reaction to 1,8-cineole (Kampranis et al. 2007). Also in *Ocimum basilicum,* the structural basis of TPS specificity was investigated through site-directed mutations in geraniol synthase. The results obtained suggested that the right half of the catalytic site plays an important role in GPP transformation, either by stabilizing the GPP substrate on the catalytic site, or by enabling its transformation into a monoterpene through an intermediate carbocation. On the other hand, the residues from the left half of the catalytic site were involved in interactions with the 3 Mg^{2+} (Fischer et al. 2013b). Two diterpene synthases, isopimaradiene synthase and levopimaradiene/abietadiene synthase, from *Picea abies* with 91% identity at the amino acid level were mutagenized to identify the specific residues that direct the different product outcomes (Keeling et al. 2008). One amino acid mutation switched the levopimaradiene/abietadiene synthase into producing isopimaradiene and sandaracopimaradiene instead of the normal products. Four mutations were sufficient to reciprocally reverse the product profiles of both paralogous enzymes. In this study, Keeling et al. (2008) demonstrated how neofunctionalization can result from relatively minor changes in protein sequence.

Many of the mutational studies demonstrate that the product specificity of terpene synthases is both dependent on the amino acid residues forming the active site cavity and the special constraints of the active site (Degenhardt et al. 2009). The mutagenesis studies highlight the complexity of predicting product profiles based on sequence similarity and show that specific mutations within the active site, or other possible mutations throughout the enzyme, can affect the functionality as well as the diversity of the products. Such flexibility is an important consideration when comparing sequences between species and studying the evolution of terpene synthase diversity (Keeling and Bohlmann 2006).

5.5. Transcriptional regulation of the monoterpene synthase genes in *in vitro* cultures

Volatile terpenes are often biosynthesized and emitted from specific plant tissues at a particular time. Some reports described the spatio-temporal expression of TPSs correlating with volatile terpene biosynthesis and emission, thus indicating a main regulation at transcriptional level (Nagegowda 2010).

In this study, semi-quantitative PCR analysis showed that the three *Tctps* genes were highly expressed in shoots but not in roots and that *Tctps2* and *Tctps5* were more expressed than *Tctps4*, since a higher number of cycles was needed for semi-quantitative PCR analysis of the latter. During the period of analysis, *Tctps2* transcripts were always present in the shoots of the five genotypes, however for *Tctps4* this was true for C, CT and SC, but not for G1 and G2 where lower transcripts were detected between 1-22 days followed by a slight increase by day 30. This dual differential gene expression ensures that the biosynthetic machinery has a continuous source of γ-terpinene and the biosynthetic related products, carvacrol and thymol, which are the main components of the essential oils. *Tctps5* transcripts were only detected, by this method, in G1 and G2 genotypes. These plants resulted from open crosses of a plant with the α-terpineol chemotype (Chapter II) and presented low amounts of α-terpineol (about 1%) in the essential oils. The absence or low expression level of these genes in the roots was expected, because the expression of terpene synthase genes is highly up-regulated and only occurs in specialized cells, such as glandular trichomes (Bohlmann et al. 1998; Tholl 2006), which only develop in the aerial parts of the plant.

To get an insight on temperature effect on Tctps activity, the enzymatic assays were performed at different temperatures (4, 21, 42ºC), suggesting this is an area to be further addressed. However, it remains to be disclosed how *Tctps* genes are regulated under different conditions. So, it would be interesting to evaluate how temperature affects *Tctps* gene expression, by growing plants at different temperatures and assessing gene expression and product formation.

6. Conclusion

This work reports the presence of three monoterpene synthase genes in *T. caespititius*, *Tctps2, Tctps5* and *Tctps4;* the latter being described for the first time. *Tctps4* encodes a γ-terpinene synthase, and appears to be an isogene of *Tctps2*. This new gene may be the result of spontaneous mutations that did not compromise protein function as shown in *in vitro* assays. However, the activity of this protein *in vivo* remains to be elucidated. *Tctps* differential expression profiles in shoot cultures was studied along the culture period and in the different chemotypes.

Mutagenic studies demonstrated that Arg-505 residue (R505G) in Tctps2 is essential for protein

stability or correct folding of the active site.

This work is just a small contribution to the full understanding of how TPS genes evolved and function. Several questions remain unanswered, such as 1) the regulation of terpene synthases and other terpenoid-related enzymes, 2) the signaling pathways, 3) the promoter elements involved in regulation and 4) the possible post-translational regulation of the enzymes.

T. caespititius in vitro cultures proved to be a good experimental model to further investigate terpene metabolism and elucidate many of these unanswered questions.

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Chapter IV

Influence of culture media and fungal extracts on monoterpene biosynthesis in *Thymus caespititius*

Part of the results presented in this chapter were previously published.

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1. Abstract

The effect of culture media composition and fungal extracts on essential oils composition and on the expression of terpene synthase genes was evaluated in *T. caespititius* shoot cultures. Nutrient composition influenced quantitatively the essential oils content. A decrease in the acetate compounds in the essential oils was observed in SH medium as compared with MS. The accumulation of terpene synthase (*Tctps*) transcripts was higher in all genotypes grown on SH, except in sabinene/carvacrol (SC) genotype. *B. cinerea* extracts highly induced *Tctps* gene expression in the first hours after treatment in SC genotype, while in carvacrol/thymol (CT) only a slight increase was observed. *Tctps* down-regulation observed during the next time points could result from reallocation of energy resources to plants' vital functions. Fungal extracts did not affect qualitatively and quantitatively the CT essential oils, however in SC, the sabinene relative content decreased and this was inversely related to the increase in the oil of two antifungal compounds, carvacrol and thymol. *T. caespititius in vitro* cultures revealed to be a good system to study terpene metabolism study.

2. Introduction

As mentioned in Chapters I and II, *Thymus caespititius* is an endemic species from the NW Iberian Peninsula and the Azores and Madeira archipelagos (Santos et al. 2005), with seven well defined chemotypes, carvacrol, thymol, α-terpineol, sabinene, carvacrol/α terpineol, α terpineol/T cadinol and carvacrol/thymol (Salgueiro 2006; Figueiredo et al. 2008a; Trindade et al. 2008).

In general, terpenes (mono and sesquiterpenes) are the predominant constituents of essential oils, but other chemical groups, such as phenylpropanoids, are also common (Sangwan et al. 2001; Figueiredo et al. 2008b). As referred in Chapters I and III, terpenes are extremely variable in chemical structure (approximately 65,000 have been identified), however, they share a common biosynthetic pathway, and the classification of terpenes is based on the number of common five-carbon (C5) isoprene units in the skeletal structure (Degenhardt et al. 2009; Yu and Utsumi 2009; Koksal et al. 2011; Oldfield and Lin 2012). Most of the terpenes are derived from structures synthesized by terpene synthases (TPSs) from one of three common prenyl diphosphate precursors, geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) (Keeling and Bohlmann 2006). Volatile terpenes are often biosynthesized and emitted from specific plant tissues at a particular time. Previous reports described that the spatio-temporal expression of TPSs correlated with terpene biosynthesis and emission, indicating that terpene biosynthesis was regulated at transcriptional level (Tholl 2006; Nagegowda 2010). Terpenes are involved in several ecological

and physiological functions on the basis of the differential expression profiles of terpene synthase genes observed during plant development and in response to biotic and abiotic environmental factors (Facchini and Chappell 1992; Dudareva et al. 2004, Tholl 2006; Yu and Utsumi 2009).

The development and adoption of tissue culture methods has provided means for rapid propagation of selected genotypes and chemotypes of several medicinal and aromatic plants (Rout et al. 2000). *In vitro* techniques provide model systems for the study of production, accumulation and metabolism of important metabolites, avoiding plant collection from their natural habitat (Karuppusamy 2009; Ncube et al. 2011).

Optimization of medium nutrients is important to increase the productivity of particular secondary metabolites (Saito and Mizukami 2002; Luczkiewicz 2008). The most commonly used culture media are based on the established formulations defined by Gamborg et al. (1968), Heller (1953), Linsmaier and Skoog (1965), Murashige and Skoog (1962), Schenk and Hildebrandt (1972) and White (1943), among others. Nitrogen, phosphate, potassium, and sucrose, as well as the NH_4^+/NO_3^- ratio, are important factors for secondary metabolite production (Coste et al. 2011).

In vitro plant secondary metabolite production can be enhanced through the application of a variety of elicitors to tissue culture systems (Vasconsuelo and Boland 2007; Rhee et al. 2010). Elicitors are signal molecules that activate multiple signaling events and lead to activation or *de novo* biosynthesis of transcription factors, which regulate the expression of biosynthetic genes involved in plant secondary metabolism (Zhao et al. 2005; Yamaner et al. 2013). Fungal elicitors, mostly derived from the cell walls of pathogens, are known to induce *de novo* synthesis of antimicrobial compounds, the phytoalexins, which are involved in the defense against phytopathogenic microorganisms (Szabo et al. 1999).

Among the phytopathogenic fungi, the genus *Botrytis* is particularly important, because species of this genus are widespread and cause severe damage to some agricultural crops. In particular, the yield losses of fruit and vegetable crops may be as high as 45–60% (Perkovskaya et al. 2004). *B. cinerea* is a nonspecific fungus that infects a wide range of host plants (up to 200 plant species) (Perkovskaya et al. 2004). These authors verified that chemically different biotic elicitors (protein and carbohydrate fractions) derived from *B. cinerea* induced reactive oxygen species and phytoalexin formation in onion cells. Two distinct elicitors, botrycin and cinerein, derived from *B. cinerea* induced the expression of defense and resistance genes, as well as some genes from secondary metabolism (Repka 2006). In grapevine cells, *B. cinerea* infection drastically enhanced the stilbene synthase leading to high stilbene formation (Liswidowati et al. 1991).

In order to understand the regulation of terpene synthase genes and their influence on essential oil composition, the effect of different culture media on the gene expression and on the essential oil

composition in five *T. caespititius* genotypes grown *in vitro* was studied. The effect of fungal extracts was included in the study using two selected genotypes.

3. Material and Methods

3.1. Plant material and routine subculture

Clonally micropropagated shoots from three *T. caespititius* genotypes belonging to different chemotypes, carvacrol (C), carvacrol/thymol (CT), sabinene/carvacrol (SC) were established from axillary buds collected from field-grown plants and maintained *in vitro* in MS medium (Murashige and Skoog, 1962) according to Chapter II. In addition, seedlings with an undefined chemotype (G1 and G2) were obtained from an α-terpineol plant and cultivated *in vitro* under the same conditions.

Routine culture conditions were performed every 4 weeks, as previously reported in Chapter II. Elongated shoots fully established *in vitro* were used as experimental material for subsequent experiments.

3.2. Culture media assay

The five genotypes established *in vitro* on MS were transferred to basal SH (Schenk and Hildebrandt 1972) medium with the same supplementation used in the routine procedure (control MS medium).

The samples were collected from shoots at the end of the subculture for essential oils isolation and RNA extraction at 6^{th}, 8^{th}, 10^{th} and 12^{th} subcultures after *in vitro* transfer to SH medium, as well in the corresponding period for plants grown on MS. For RNA extraction all samples were frozen in liquid nitrogen immediately after collection and stored at −80ºC until processing. A pooled sample was obtained using young leaves from five *in vitro* shoots, to minimize individual differences.

3.3. *Botrytis cinerea* culture and experiment

Botrytis cinerea Pers. (10.165, MUM, Braga, Portugal), was grown on potato dextrose agar (PDA) medium for 8 days in the dark at 24ºC, for profuse sporulation. The sporulated mycelium was grounded in liquid nitrogen, and added at 4 $g.L^{-1}$ to MS medium supplemented with 0.4 $mg.L^{-1}$ BA and 0.1 $mg.L^{-1}$ IBA, followed by routine medium sterilization in autoclave.

Only two *T. caespititius* genotypes (CT and SC) established *in vitro* were transferred to MS medium with fungal extracts (MS+FE) and grown for 30 days. Growing conditions were the same described for routine cultures. The samples for RNA isolation consisted of young leaves, collected from shoots at 0,

3, 6, 12 hours and 1, 4, 8, 15 and 30 days. For essential oils isolation, samples were collected only at the end of the subculture. The experiment was performed twice.

3.4. Statistical Analysis

Growth rate was evaluated considering biomass increase and *in vitro* proliferation rates. Biomass increase was calculated on a fresh weight basis (fw), while proliferation rate was assessed by counting the shoots number in each cluster. Initial average shoot weight was 80 mg. Mean values ± standard errors (SE) were obtained from fifteen independent replicates and the experiments were performed twice. The statistical significance of data obtained from the five genotypes was determined by one-way analysis of variance (ANOVA) for the two sets of data. All statistical analyses were performed using Microsoft Excel 2010.

3.5. Essential oils isolation

Essential oils were isolated from 3-5 g of shoots growing on MS or SH media. After 1-year establishment on MS, sampling was performed at the 6^{th}, 8^{th}, 10^{th} and 12^{th} subcultures after transfer to SH, and on the corresponding periods for the cultures maintained on MS (control). For the MS+FE medium, shoots were collected after 30 days. Essential oils were isolated according to described in Chapter II. Two replicates were used per experiment. The data was calculated as mean values of each experimental point.

3.6. Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatographic and GC-MS analyses of the essential oils were performed as described in Chapter II. The percentage composition of the oils was calculated as mean values of two injections from each oil, without using correction factors. Two oils replicates were used per experiment. The data was calculated as mean values of each experimental point.

3.7. Total RNA extraction and cDNA preparation

Young leaves (approximately 100 mg) were excised from *in vitro* shoot-cultured *T. caespititius* plantlets, grounded in liquid nitrogen with mortar and pestle, and the total RNA was extracted using TriReagent® Solution (Ambion®) according to the manufacturer's instructions. Quality and concentration of total RNA were assessed by NanoDrop1000 spectrophotometer (Thermo Scientific) at 260 nm.

For cDNA synthesis, 1 µg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions.

3.8. Semi-quantitative RT-PCR analysis for gene expression profiling

For expression profile analysis, three *Tctps* genes previously isolated and characterized in *T. caespititius* (*Tctps2, Tctps4* and *Tctps5*) (Chapter III, Lima et al. 2013) were analyzed by semi-quantitative RT-PCR. A fragment with approximately 500 bp of the respective cDNAs was amplified using GoTaq DNA polymerase (Promega) and the primer pairs TPS2_1Fw and TPS2_4Rv used for *Tctps2*, TPS4_1Fw and TPS4_4Rv for *Tctps4* and TPS5_1Fw and TPS5_1Rv for *Tctps5* (Table 1). The *Thymus elongation factor 1-alpha gene* (*ef-1α*) was amplified as a control of constitutive expression, to estimate if equal amounts of cDNA were used among samples. The primers used were designed from *Origanum vulgare* (Crocoll et al. 2010). Reactions were performed on a T-Gradient thermocycler (Biometra) with the following cycles: 95ºC for 5 min, followed by 28 or 35 cycles (28 cycles for *Tctps2* and *Tctps5*, and 35 cycles for *Tctps4*) at 92ºC for 1 min, 60ºC for 45 s and 72ºC for 1 min, followed by a 5 min final extension step at 72ºC. PCR reactions were repeated with two independent biological replicates.

Table 1. Oligonucleotide sequences used for semi-quantitative PCRs.

	Primer ID	Primer sequence (5'-3')
Tctps2	TPS2_1Fw	ATGGCTACCCTTAGCATGCAAG
	TPS2_4Rv	CCAAGAGATGTGAAGTACAAATCCCC
Tctps4	TPS4_1Fw	ATGGAAGGTTCAAAACCAGC
	TPS4_4Rv	CTCATCATCGATTCCTTTTTCATC
Tctps5	TPS5_1Fw	ATGTCTACCATTAGCATACATCATGTGGGC
	TPS5_1Rv	GATGCTTCGTAGAGTTGCAGCGTTGC
Elongation factor 1-α	EF_1Fw	CAACATATCTGAGCCCAAGAGG
	EF_1Rv	GCTCCTTTCCAGACCTCCTATC

3.9. Quantification of transcripts

Relative abundance of transcripts was determined through quantitative analysis of the digital gel images, using the GeneTools (Syngene, UK) software, after normalizing mRNA levels against the housekeeping gene *ef-1α*. *Tctps2* and *Tctps5* gene expression was determined by semi-quantitative RT-PCR analysis using 28 cycles and 35 cycles for *Tctps4*. The transcript levels in each sample were calculated relative to the control transcript level (0 time point, starting point with constitutive gene expression, without any stress condition; or plants grown in MS medium), which was set to 1. In the case of the *B. cinerea* infection kinetics, the expression reported is the ratio between transcript

abundance in the infected and the non-infected tissue sample at the same time point. For gene expression comparison between the different genotypes, the expression reported is the ratio between *Tctps* transcript abundance and *ef-1α* at the same time point.

4. Results

4.1. Effect of the culture media formulation in shoot cultures

4.1.1. Shoot proliferation

The five genotypes (C, CT, SC, G1 and G2) were established *in vitro* on MS medium (Fig. 1B, 1C, 1F). After complete establishment, some clonal shoots from each genotype were transferred to SH medium (Fig. 1D, 1G).

As mentioned in Chapter II, plant clusters with 60-100 mg and 3 to 6 shoots were used during the subculture period. Shoot growth was evaluated by fresh weight increase on both media. Fresh weight increased between 3.1- and 6.1-fold in MS shoot cultures while on SH it increased between 2.2- and 4.9-fold. The proliferation rate (number of new shoots produced in each culture) was similar for both culture media, varying from 1.7- to 2.9-fold for MS shoot cultures and from 1.7- to 2.8- for plants grown on SH medium (Table 2).

Table 2. Evaluation of shoot proliferation rate in *in vitro* grown *T. caespititius* using two different methods – fresh weight increase and shoots number - after a 30 day period on MS, SH and MS+FE media supplemented with 0.4 mg.L-1 BA and 0.1 mg.L-1 IBA.

Genotype	Culture media	Mean fresh weight	Mean number of shoots
C	MS	3.3±0.4 a	2.2±0.3 a
	SH	2.9±0.4 a	2.3±0.3 a
CT	MS	4.5±0.8 a,b	2.5±0.4 a,b
	SH	4.9±0.7 b	2.8±0.4 a,b
	MS+FE	2.1±0.2[c]	1.3±0.1 c
SC	MS	6.1±0.7 b,d	2.7±0.2 a,b,d
	SH	3.5±0.3 a,e	2.1±0.3 a,d
	MS+FE	3.2±0.4 f	1.9±0.1 e
G1	MS	4.5±0.4 a,b,g	2.9±0.4 a,b,d,f
	SH	2.2±0.2 a,h	1.7±0.2 a,d,g
G2	MS	3.1±0.3 a,b,i	1.7±0.2 a,b,h
	SH	2.3±0.2 a,h,j	2.1±0.3 a,b,d,g,h

Data indicate mean ± SE (n = 10), and letters that are different within the same column indicate statistical differences ($P \leq 0.05$).

The G2 genotype showed the lowest proliferation rate. The growth rate in C and CT genotypes in plants grown on MS and SH media was not statistically significant (Table 2). The differences in the proliferation rate were only statistically significant for G1 genotype (Table 2).

In vitro shoots were morphologically similar to field grown plants (Fig. 1A), although with more elongated internodes. Spontaneous root formation occurred in all plants (Fig. 1C, 1D, 1F, 1G) however in SH medium root development was more evident. Roots were discarded upon subculture.

Figure 1. *Thymus caespititius* field-grown plants (A) and *in vitro* shoot cultures (B-H). (B) *In vitro* cultures growing in transparent aerated containers and (C-H) detail of the proliferation of multiple shoots from *in vitro* derived nodal explants, maintained on MS (C, F), SH (D, G) and MS with fungal extract (E, H) media, supplemented with 0.4 $mg.L^{-1}$ BA and 0.1 $mg.L^{-1}$ IBA. (F-H) Example of the spontaneous root formation observed in all shoot cultures (Bars = 1 cm).

4.1.2. Essential oils

The essential oils isolated from the five genotypes had a strong odour and a yellow colour. The oil yields ranged from 0.3 to 1.2% (v/fw; $mL.g^{-1}$) in plants grown in MS medium and 0.3 to 3.4% (v/fw; $mL.g^{-1}$) in plants grown on SH medium (Table 3). The oil yields were higher in all genotypes grown in SH, except for C genotype, where no major differences were observed.

Table 3. Minimum and maximum percentage range (≥ 3%) of components identified in the essential oils isolated from five *in vitro T. caespititius* genotypes cultivated on MS and SH media supplemented with 0.4 mg.L-1 BA and 0.1 mg.L-1 IBA (sampled at the 6th, 8th, 10th, and 12th subcultures after *in vitro* establishment in SH medium).

		C plants				CT plants				SC plants				G1 plants				G2 plants			
		MS		SH		MS		SH		MS		SH		MS		SH		MS		SH	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Components	**RI**																				
α-Thujene	924	1.1	3.9	2.3	4.1	2.1	3.5	2.4	3.1	1.9	3.3	2.4	3.3	1.3	2.2	0.9	3.0	1.4	3.0	1.3	2.3
Sabinene	958	0.1	0.3	0.2	0.3	0.2	0.4	0.3	0.4	34.6	44.3	41.5	44.8	0.4	0.8	0.7	1.3	0.1	0.5	0.7	1.3
p-Cymene	1003	2.3	4.8	4.9	5.9	5.2	7.6	6.1	8.6	4.1	4.8	4.4	6.5	7.1	11.3	9.6	20.7	12.4	20.7	27.7	33.6
γ-Terpinene	1035	1.2	4.7	4.4	5.3	4.4	6.8	8.1	9.5	3.4	4.2	3.6	4.1	4.1	6.8	6.1	15.2	8.5	10.2	15.5	20.9
α-Terpineol	1159	t	t	t	t	t	t	t	t	t	t	t	t	0.1	0.7	1.5	3.1	t	0.8	1.5	2.3
Carvacrol methyl ether	1224	t	t	t	t	t	t	t	t	t	t	t	t	10.7	13.6	4.6	8.2	11.3	17.3	3.6	8.0
Thymol	1275	t	t	t	t	13.8	22.5	19.4	22.6	8.3	10.8	9.9	11.2	11.7	13.7	11.0	13.3	12.1	17.5	3.6	6.7
Carvacrol	1286	25.2	46.4	55.8	59.6	12.7	22.6	23.6	27.6	11.0	14.9	16.8	18.5	4.3	7.5	3.6	7.1	4.0	7.9	0.6	2.3
Thymyl acetate	1330					10.2	18.0	7.6	10.5	3.3	6.3	0.5	1.3	3.9	6.9	1.6	4.2	3.1	7.5	0.2	0.6
Carvacryl acetate	1348	23.0	44.8	9.1	12.5	13.4	26.0	9.5	13.3	5.2	9.3	1.0	1.9	1.0	2.9	0.4	1.9	0.6	1.6	t	0.1
Elemol	1530	0.2	0.6	0.3	0.3	t	0.1	0.1	0.2	0.2	0.3	0.3	0.3	4.8	5.7	3.4	5.6	1.9	2.9	2.7	3.3
UIA Thymus	1597	0.8	1.5	0.9	1.0	0.2	0.5	0.3	0.4	0.6	1.1	0.8	1.0	1.2	1.7	1.0	1.5	0.6	1.1	0.8	1.1
UIB Thymus	1609	3.8	6.8	3.6	4.4	1.0	2.1	1.3	1.7	2.8	4.8	3.3	4.6	6.4	9.0	4.4	7.5	3.0	4.7	3.3	4.7
τ-Cadinol	1616	1.1	2.3	1.0	1.3	0.6	1.3	0.7	0.9	0.5	1.0	0.6	0.8	7.2	9.4	4.2	9.3	1.5	2.3	1.9	2.4
UIC Thymus	1648	1.5	2.8	1.5	1.8	0.3	0.8	0.5	0.7	1.1	4.6	1.4	1.8	2.3	3.4	1.7	2.9	1.2	1.7	1.3	1.9
UID Thymus	1662	0.2	0.4	0.2	0.5	0.1	0.3	0.3	0.5	t	0.2	0.2	0.4	0.5	0.8	0.8	1.2	1.4	2.5	2.6	3.5
% Identification		81.4	91.8	90.2	91.8	90.3	95.1	91.8	96.1	90.8	95.3	92.1	93.9	78.7	84.0	80.6	86.6	84.1	91.5	82.0	87.4
Grouped Components																					
Monoterpene hydrocarbons		7.5	17.4	16.2	20.3	15.4	23.5	22.1	26.6	46.7	58.4	57.4	59.1	15.9	24.4	20.4	47.5	30.2	43.3	53.7	60.5
Oxygen-containing monoterpenes		66.8	72.6	66.7	69.5	69.0	71.9	66.2	68.6	30.8	38.8	28.6	32.4	35.5	42.9	24.4	36.5	37.9	47.3	11.3	19.0
Sesquiterpene hydrocarbons		1.2	2.1	1.1	1.3	0.6	1.2	0.6	0.9	0.8	1.4	0.9	1.4	6.0	7.3	4.8	7.3	3.1	4.8	4.3	6.4
Oxygen-containing sesquiterpenes		2.9	5.1	2.8	3.3	1.4	3.1	2.0	2.6	2.3	3.9	2.9	3.6	14.7	18.2	9.6	18.0	5.3	7.6	6.6	8.5
Fatty acids		t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Others		t	t	t	0.1	t	0.2	t	t	t	t	t	t	0.4	0.6	0.3	0.4	0.6	1.1	0.3	0.4
Oil yield (%)		0.3	1.2	0.7	1.5	0.3	1.0	0.6	2.2	0.3	0.9	0.8	2.4	0.2	0.7	0.3	1.4	0.2	0.7	0.7	1.5
Biosynthetic pathways																					
Carvarol/Thymol pathway		71.4	79.1	77.6	79.2	80.7	84.5	80.1	84.4	38.8	47.0	37.2	40.7	47.8	55.6	47.2	57.1	65.2	68.9	56.7	64.1
Sabinene pathway		0.1	0.3	0.2	0.3	0.2	0.4	0.3	0.4	34.6	44.3	41.5	44.8	0.4	0.8	0.7	1.3	0.1	0.5	0.7	1.3
α-Terpineol pathway		t	t	t	t	t	t	t	t	t	t	t	t	0.1	0.7	1.5	3.1	t	0.8	1.5	2.3

RI - Retention Index relative to C_9-C_{20} *n*-alkanes on the DB-1 column; t - trace (<0.05); UIA, UIB, UIC, UID Thymus are four unidentified components present in the essential oils.

The essential oil isolated from each sample was a complex mixture in which 63 components were identified, representing ≥ 81% of the total volatiles. The main components (≥ 3%) identified in the essential oils are listed in Table 3 in the order of their elution on the DB-1 column, arranged according to the plant genotype with the lowest and the highest percentages found for each component in each oil type.

The monoterpene fraction was dominant in all samples analyzed, ranging between 53-94%, while the sesquiterpene fraction ranged from 2-25% (Table 3). Carvacrol (25-60%) and carvacryl acetate (9-45%) were the main components of C (carvacrol chemotype) genotype (Table 3, Fig. 2A). Plants grown on SH medium had less relative amount of carvacryl acetate (9-13%) than plants grown on MS medium (23-45%). The essential oils from the CT shoot cultures (carvacrol/thymol chemotype) were dominated not only by carvacrol (13-28%) and thymol (14-23%), but also by carvacryl acetate (10-26%) and thymyl acetate (8-18%) (Table 3, Fig. 2B). In this genotype the acetate compounds (thymyl and carvacryl acetate) also showed a relative amount decrease. Sabinene (35-45%), carvacrol (11-19%) and thymol (8-11%) were the main components of SC *in vitro* shoots, sabinene/carvacrol chemotype (Table 3, Fig. 2C). A decrease in carvacryl acetate and thymyl acetate relative content was observed in oils isolated from shoots grown on SH medium (respectively 5-9% in MS vs 1-2% in SH; 3-6% in MS vs 1-2% in SH).

The main components identified in the G1 and G2 essential oils were *p*-cymene (7-34%), γ-terpinene (4-21%), thymol (4-18%) and carvacrol methyl ether (4-17%). In SH, the relative amount of carvacrol methyl ether (4-8%) decreased, while *p*-cymene (10-34%) and γ-terpinene (6-21%) showed an inverse tendency (Table 3, Figs. 2D, 2E). In G2 genotype a decrease in thymol (4-7%) relative amount was also observed (Table 3, Fig. 2E).

The main quantitative differences observed between the oils from shoots grown in the two culture media were related to the acetate compounds. Considering the monoterpenes, no major differences were detected when the compounds were grouped according to their biosynthetic pathways (Table 3).

4.1.3. Terpene synthase gene expression

Terpene synthase (*Tctps2, Tctps4* and *Tctps5*) gene expression was determined by semi-quantitative RT-PCR analysis. Total RNAs extracted from *T. caespititius* shoots grown on two different media, MS and SH, were analyzed. Constitutive expression of the *ef-1α* gene was used for normalization of band intensities using GeneTools software.

The optimized semi-quantitative PCRs were performed following 28 cycles for *Tctps2* and *Tctps5* and 35 cycles for *Tctps4*, revealing the different expression levels of these genes (Fig. 3). *Tctps2* was expressed at higher basal level in plants grown on SH medium, except for SC genotype where the

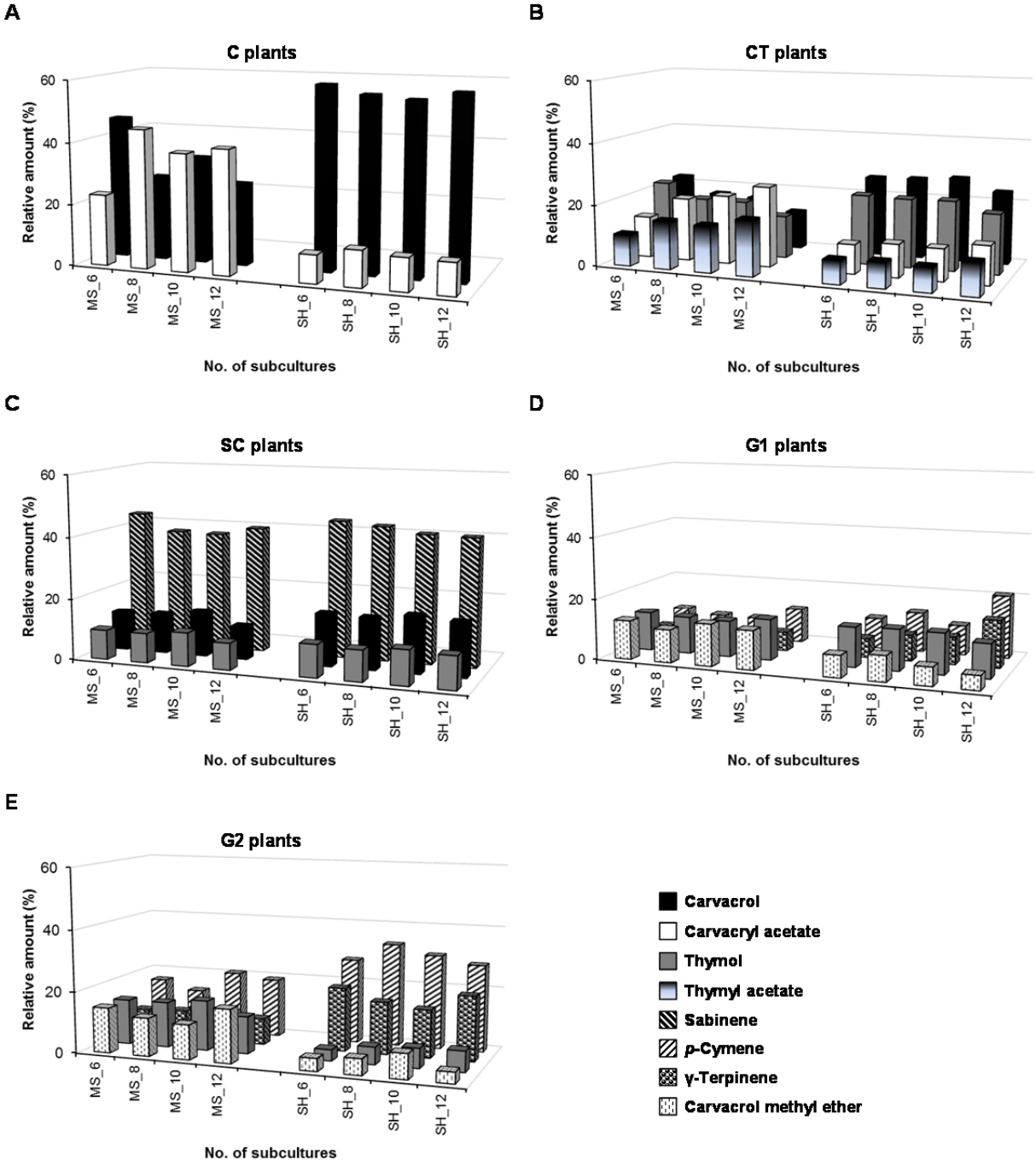

Figure 2. Variation in the relative amount of the main components of *in vitro T. caespititius* essential oils, isolated from shoot cultures on MS and SH media supplemented with 0.4 mg.L^{-1} BA and 0.1 mg.L^{-1} IBA at 6th, 8th, 10th, 11th and 12th subcultures after culture establishment in SH medium. A) C genotype. B) CT genotype. C) SC genotype. D) G1 genotype. E) G2 genotype. Standard deviation never exceeded 1% and for clarity SD bars were not included in the graphs.

expression was higher in plants grown on MS medium and for the G1 genotype where the expression was similar between the two conditions (Fig. 3). Similarly as for *Tctps2*, *Tctps4* expression levels were higher in plants grown on SH medium, except for the SC genotype. This gene was less expressed in G1 and G2 than in the other genotypes. *Tctps5* gene expression was only detected in G1 and G2 and it was higher in plants grown on SH medium (Fig. 3). As expected, *Tctps5* gene expression was only detected in shoot cultures with higher relative amount of α-terpineol.

The transcript levels in each sample were calculated relative to the control MS, where the transcript levels were set to 1. Expression levels of the three *Tctps* genes were higher in shoots grown on SH medium than in MS, except for SC shoots (Fig. 3 and 4). Transcript levels of *Tctps2* and *Tctps4* increased between 1.2- to 3.3-fold and 1.1- to 5.8-fold, respectively. However, in SC shoots transcript level decrease was observed in both genes on SH, ranging from 2.4- to 1.7-fold (Fig. 4). *Tctps5* specific transcripts were induced up to 11.2-fold (Fig. 4A) in G1 and G2 genotypes grown on SH.

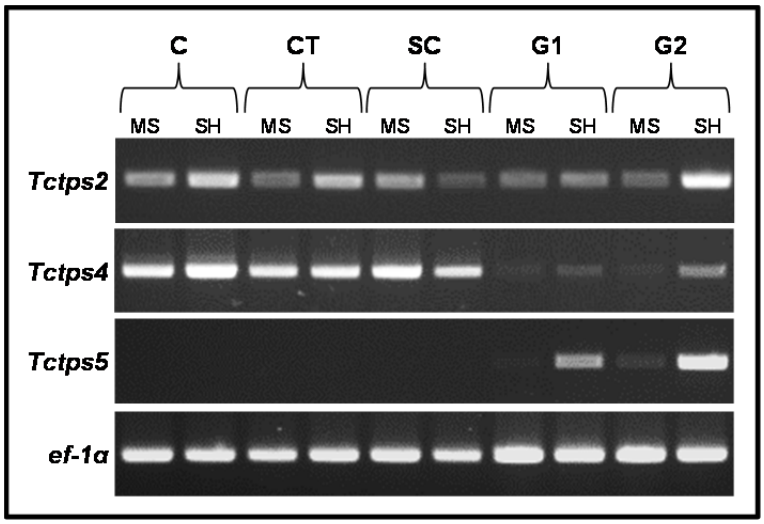

Figure 3. Semi-quantitative RT-PCR of terpene synthase transcripts (*Tctps2, Tctps4,* Tctps5) in five different plant genotypes cultivated in two culture media (MS and SH). The *Thymus elongation factor 1-α* gene was used as control. The experiment was repeated twice.

Expression levels of the three *Tctps* genes were compared between the five genotypes, grown in the two culture media (Fig. 4B). *Tctps* relative expression levels in each sample were calculated as the ratio between *Tctps* and *ef-1α* transcripts abundance. Higher amount of *Tctps2* transcripts was observed in C genotype than in the other genotypes; however, the G2 genotype grown on SH was the exception, showing higher expression of this gene (Fig. 4B). The C plants grown on both media also showed higher *Tctps4* expression than the other genotypes, except the SC plants grown on MS, where the expression of this gene was slightly higher. In G1 and G2 this gene was downregulated in comparison to the others four genotypes. *Tctps5* expression in plants grown on MS was extremely

reduced, but a very strong increase in *Tctps5* transcript accumulation was observed on SH medium, reaching values of 0.5 and 1 in G1 and G2 respectively (Fig. 4B).

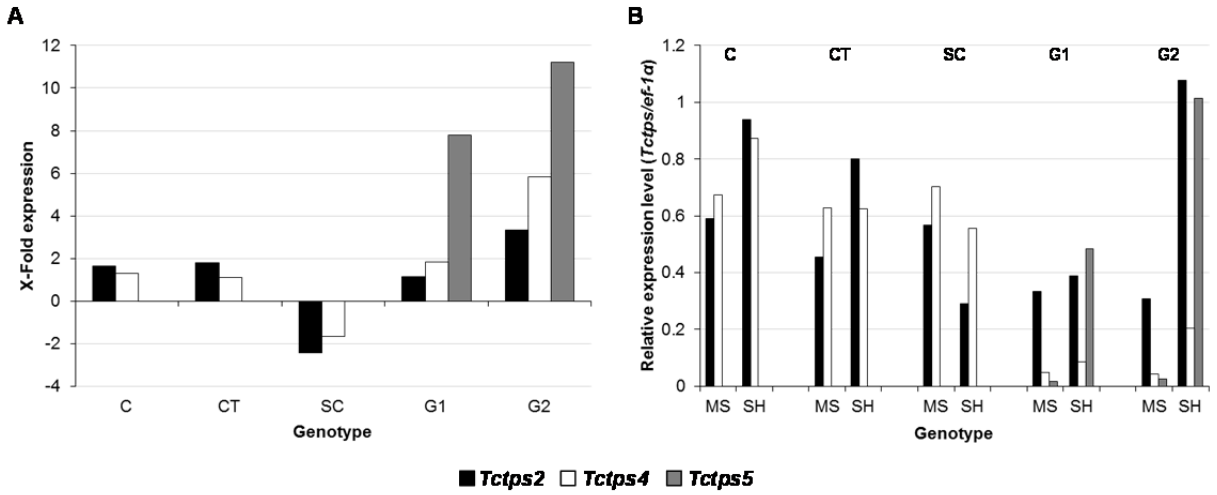

Figure 4. Quantification using the GeneTools software of RT-PCR products of terpene synthase genes in five different plant genotypes cultivated in two culture media (MS and SH). A) x-Fold expression was calculated by dividing the normalized band intensity values of plants grown on SH medium by the values of plants grown on MS medium (control). B) The values presented were normalized relative to the housekeeping gene (*ef-1α*), equal 1, allowing the comparison between genotypes and culture conditions.

4.2. Fungal extracts affect differently the two genotypes

4.2.1. Shoot proliferation

Two genotypes (CT and SC) fully established on MS medium (Fig. 1C and 1F) were transferred to MS medium supplemented with *Botrytis cinerea* extracts, MS+FE, for 30 days. During this period shoot cultures started to show some phenolic exudare and oxidation, necrotic tissues and reduced growth rate (Fig. 1E, 1H). After the 30 days period, the CT plants died. However, the SC genotype was maintained in this medium (MS+FE) for another 30 days and was able to recover and proliferate (data not shown).

The growth and proliferation of the shoot cultures were lower in MS+FE as compared to control MS, with statistically significant differences, as shown in Table 1. Evaluation of shoot growth on MS+FE media revealed that fresh weight increase varied between 2.1- and 2.8-fold, while the proliferation rate varied from 1.3- to 1.9-fold for CT and SC genotypes, respectively (Table 1).

4.2.2. Essential oils

The monoterpene fraction was dominant in all samples analysed from CT and SC, ranging between 72-86%, while the sesquiterpene fraction ranged from 4-9% (Table 4). In CT shoot cultures,

the fungal extract didn't affect quantitatively the chemical composition of the essential oils, and carvacryl acetate (26%), thymyl acetate (17-18%), thymol (15-16%) and carvacrol (14%) were the main components identified (Table 4).

Table 4. Main components (≥ 3%) of the essential oils isolated from two *Thymus caespititius* genotypes, CT (Carvacrol/Thymol) and SC (Sabinene/Carvacrol), grown on MS medium and MS with *Botrytis cinerea* extracts (MS+FE), supplemented with 0.4 mg.L-1 BA and 0.1 mg.L-1 IBA. The experiment was performed twice and the analysis was performed on five pooled plantlets.

		CT plants		SC plants	
		MS	**MS+FE**	**MS**	**MS+FE**
Components	**RI**				
Sabinene	958	0.2	0.2	34.6	8.1
p-Cymene	1003	4.0	5.3	4.8	2.2
γ-Terpinene	1035	4.2	3.0	3.5	1.9
Thymol	1275	15.9	15.2	10.8	13.8
Carvacrol	1286	14.3	13.8	14.9	18.3
Thymyl acetate	1330	17.4	18.4	5.3	11.1
Carvacryl acetate	1348	26.3	26.2	7.7	14.6
trans-β-Dihydroagarofuran	1489	0.7	0.7	1.9	3.0
UIB Thymus	1609	1.8	1.6	4.8	8.3
UIC Thymus	1648	0.8	0.7	1.9	3.4
% Identification		89.7	89.6	90.8	81.1
Grouped Components					
Monoterpene hydrocarbons		12.0	12.3	46.7	13.6
Oxygen-containing monoterpenes		74.1	73.8	38.8	58.1
Sesquiterpene hydrocarbons		0.9	0.9	1.4	2.7
Oxygen-containing sesquiterpenes		2.7	2.6	3.9	6.7
Fatty acids		t	t	T	t
Others		t	t	T	t
Biosynthetic pathways					
Carvacrol/Thymol pathway		82.1	81.9	47.0	61.9
Sabinene pathway		0.2	0.2	34.6	8.1

RI - Retention Index relative to C_9-C_{20} *n*-alkanes on the DB-1 column; t - trace (<0.05); UIB, UIC Thymus are two unidentified components present in the essential oils.

Sabinene (8-35%), carvacrol (15-18%) and thymol (11-14%) were the main components of the essential oils of SC *in vitro* shoots (Table 4). The percentage of sabinene decreased in SC shoot cultures on MS+FE medium while the opposite happened for carvacrol, thymol and corresponding acetate compounds.

The quantitative differences observed in the oils isolated from SC shoots grown in MS and in MS+FE reflected the major differences detected when the compounds were grouped according to their biosynthetic pathway (Table 4). The subtotal of carvacrol/thymol pathway compounds increased from

47 to 62% in oils isolated from plants grown with fungal extracts, with a concomitant reduction in the sabinene pathway (Table 4).

4.2.3. Terpene synthase gene expression

The expression patterns of *Tctps2* and *Tctps4* were analyzed at different times throughout the subculture period showing to be very similar, both in the control and stress conditions (autoclaved *B. cinerea* extracts, Fig. 5). As mentioned above, the optimized semi-quantitative PCRs were performed using 28 cycles for *Tctps2* and 35 cycles for *Tctps4*, revealing the different expression levels of these genes (Figs. 5 and 6). So, a direct comparison between both *Tctps* transcripts levels could not be performed.

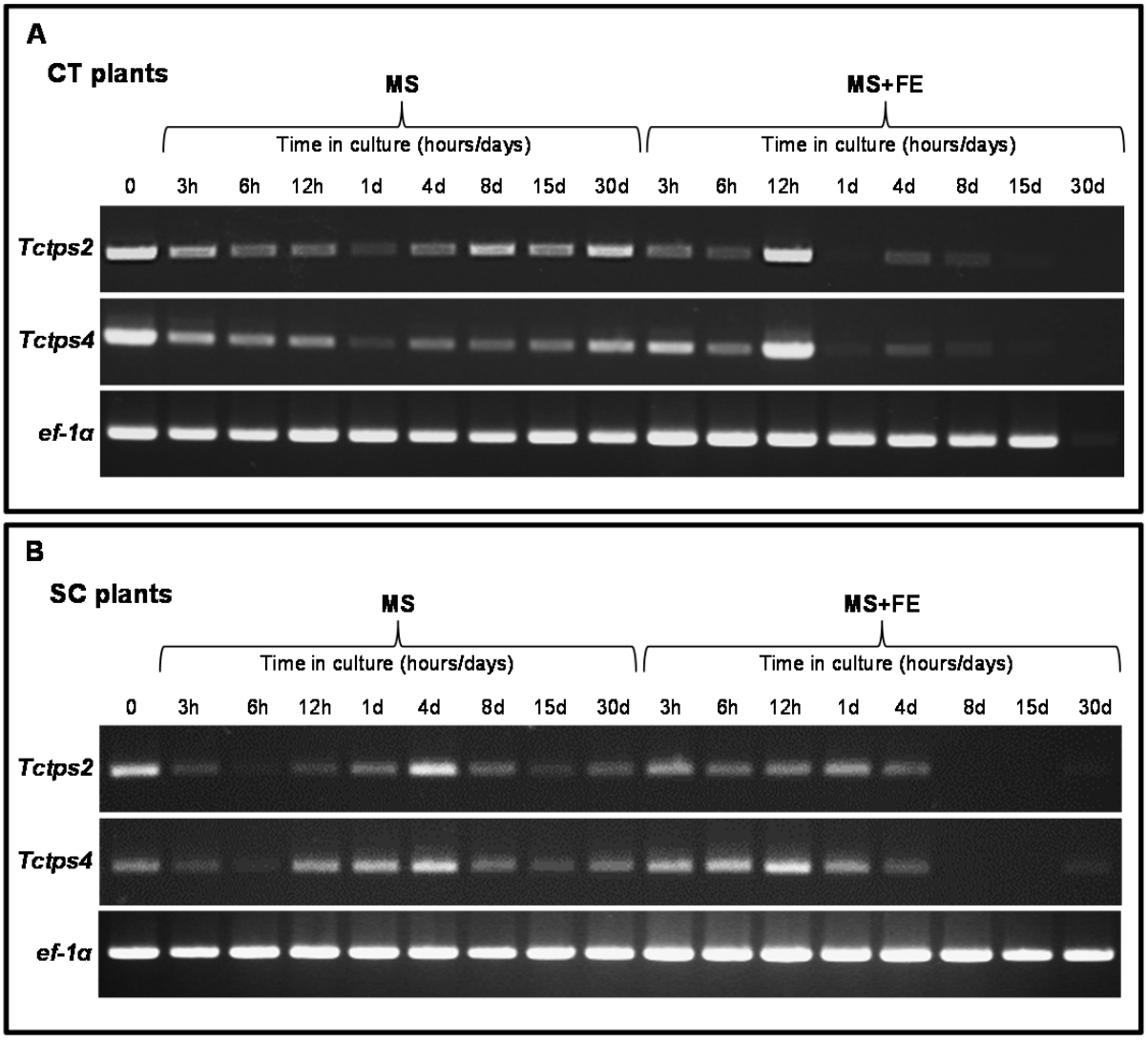

Figure 5. Semi-quantitative RT-PCR analyzes of terpene synthase transcripts (*Tctps2, Tctps4*) in two different plant genotypes, A) CT plants and B) SC plants, cultivated in MS and MS with fungal extract (MS+FE). The *Thymus elongation factor 1-α* gene was used as control. The experiment was repeated twice.

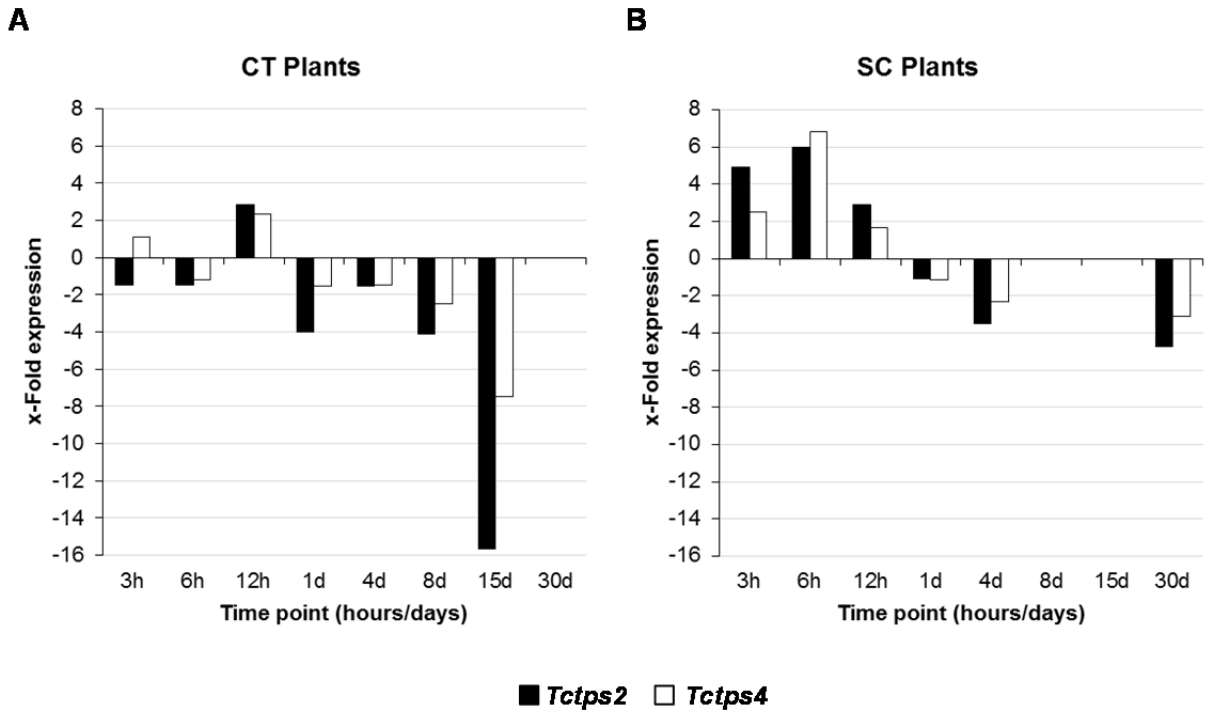

Figure 6. Quantification using the GeneTools software of RT-PCR products of terpene synthase genes (*Tctps2* and *Tctps4*) in two different plant genotypes (CT and SC) cultivated on MS and MS with fungal extract (MS+FE). A,B) x-Fold expression was calculated by dividing the normalized band intensity values of plants grown in MS+FE medium by the values of plants grown in MS medium at the same time point. The transcript levels detected prior to inoculation of fungal extracts were considered time 0 and used as control which was set to 1.

Following shoot transfer to MS+FE medium, a slight increase of expression levels of the two *Tctps* genes was observed at 12 h for CT shoots (Fig. 5A). The transcript levels increase was approximately 2.8-fold for *Tctps2* and 2.3-fold for *Tctps4* (Fig. 6A). After this time point, the expression levels decreased, about 1.5- to 15.7-fold for *Tctps2* and less for *Tctps4* (1.5- to 7.5-fold) and at 15 and 30 days the expression was too low to be detected (Figs. 5A and 6A). On the 30th day, the CT plant material grown on MS+FE was phenolized, making it impossible to isolate good quality RNA (Fig. 5A).

The transfer of the SC plants from MS to MS+FE lead to a quick accumulation of *Tctps* transcripts in the first hours, with a maximum at 6 hours after the transfer (Fig. 5B). In the first 6 h of the experiment, the transcripts of *Tctps2* and *Tctps4* were induced 6 times (Fig. 6B). Twelve hours after stress induction a slight decrease in the transcript accumulation was detected (Fig. 6B). From this time onwards a down-regulation of both *Tctps* genes was observed as compared to the control (Figs. 5B and 6B), approximately 1- to 3.5-fold in *Tctps2* transcript levels and 1- to 2.3-fold in *Tctps4*. By days 8 and 15, no expression was detected, however at the end of the subculture (day 30), a reduced expression level of both genes was registered, although smaller than in control situation (Figs. 5B and 6B).

5. Discussion

In vitro cultures represent a potential source of valuable secondary metabolites. The synthesis of phytochemicals by the tissue cultures in contrast to the synthesis *in planta* is independent of environmental conditions (Smetanska 2008). This technology was developed as a tool for studying the metabolism and production of desired secondary metabolites (Zhong 2001, Verpoorte et al. 2002).

The evolution of plant secondary compounds is often considered to be tightly associated with defense against herbivores and pathogens, either as part of their normal program of growth and development or in response to biotic and abiotic stresses (Holopainen 2004; Lattanzio et al. 2009).

The general cellular process and regulatory principle for plant secondary metabolite biosynthesis is similar to other signal perception and transduction signal in plants: an extracellular or intracellular signal is perceived by a receptor located at the plasma membrane; the elicitor signal perception initiates a signal transduction network that leads to activation or *de novo* transcription factor biosynthesis, that in turn regulate the expression of biosynthetic genes involved in plant secondary metabolism (Zhao et al. 2005).

An elicitor may be defined as a substance that can induce physiological changes. When introduced in small concentrations into a living cell system it can trigger physiological and morphological responses, like initiating or improving the biosynthesis of specific compounds (Zhao et al. 2005; Namdeo 2007). The induction mechanism of elicitors is generally regarded as inducing the expression of defense-related genes and activating defense-related secondary metabolic pathways (Qian et al. 2006). It may include abiotic elicitors such as metal ions and inorganic compounds, and biotic elicitors from fungi, bacteria, viruses or herbivores, plant cell wall components, as well as chemicals that are released at the attack site by plants upon pathogen or herbivore attack (Zhao et al. 2005). When detecting any elicitor/stress, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenols and hypersensitive death of plant cell (Smetanska 2008). Both biotic and abiotic stresses induce in plants a chain of events: signal recognition, the formation of second messengers, changes in intracellular ion fluxes (such as Ca^{2+}), the accumulation of reactive oxygen species (oxidative stress), phosphorylation of certain proteins, and transcription of the genes required for the synthesis of abscisic acid (ABA), ethylene, jasmonate, salicylate, and other compounds (Xiong et al. 2002; Dimitriev 2003, Ryabushkina 2005). These physiologically active compounds determine successive series of metabolic changes that result in the accumulation of metabolites facilitating the plant adaptation (Ryabushkina 2005). Elicitation is a very complex process and depends on many factors such as elicitor concentration, growth stage of the culture at the time of elicitor addition and contact time with the elicitor (Namdeo

2007). Elicitors provide important clues to understanding the molecular basis of the transducing pathway through which exogenous signals lead to secondary product biosynthesis (Saito and Mizukami 2002).

Many elicitors (essentially pathogens) may act as avirulence determinants of a plant genetic system that refers to a general response of gene-for-gene resistance in plant innate immunity, in which plant resistance genes (*R* genes) confer resistance to pathogens with a matching avirulence (*avr*) gene by specific recognition events. Elicitors or avirulence determinants must be recognized by plant receptors or R proteins localized in several places in the cell before initiating signaling pathways, which lead to defense reactions such as synthesis of pathogenesis-related proteins, or defense secondary metabolites (Taiz and Zeiger 2010).

5.1. Effect of the culture media formulation in shoot cultures

Plant secondary compounds normally accumulate *in vitro* at the later stages of the growth cycle, when growth slows down and excess carbohydrate and nitrogen can be redirected into secondary pathways (Collin 2001). The media composition can be optimized to promote intensive biomass increase as well as to accumulate desired metabolites, for instance by playing with the concentration and nature of the inorganic nitrogen and phosphate (Collin 2001; Matkowski 2008).

In *T. caespititius* cultures, the multiplication rate (evaluated as shoot proliferation) was similar between the two culture media and between genotypes, except for G1, that showed lower multiplication in SH. However, when comparing the fresh weights, differences were observed in some genotypes, indicating biomass growth, although the hypothesis that this only represents higher water content in the tissues cannot be discarded since the dry weight was not measured.

The different nutrient composition of the two culture media, MS and SH, did not have a qualitative effect on the chemical composition of the essential oils. Essential oil homogeneity in response to alterations in nutrient supply may indicate a strong genetic control of secondary metabolism, as reported in *Phlegmariurus squarrosus* (Ma and Gang 2008) and in *Salvia stenophylla* (Musarurwa et al. 2012).

As previously mentioned, *Tctps2* and *Tctps4* encode γ-terpinene synthase, while *Tctps5* encodes α-terpineol synthase (Chapter III, Lima et al. 2013). The higher *Tctps2* and *Tctps4* gene expression in plants grown on SH medium did not reflect a relative increase in γ-terpinene or the biosynthetic related compounds (carvacrol/thymol biosynthetic pathway). Instead, it could be related to oil yield, slightly higher in plants grown on SH medium. In SC genotype the decreased expression of both genes was related to volatile profile since a minor decrease in the subtotal of the carvacrol/thymol biosynthetic pathway was observed.

Tctps5 expression level was correlated with the α-terpineol content in the essential oils and its expression was higher in G1 and G2 shoots grown on SH medium. In C, CT and SC shoot cultures, only trace amounts of α-terpineol were detected in the essential oils, accompanied by a very low, or even absent, expression of *Tctps5* gene.

Among the factors that are important for secondary metabolite production, nitrogen, phosphate and potassium, as well as the NH_4^+/NO_3^- ratio have been reported (Coste et al. 2011). The use of a culture medium with a low N concentration and modified NH_4^+/NO_3^- ratio could lead to increased secondary metabolite production (Coste et al. 2011). Therefore, the SH medium was tested, due to its lower levels of total nitrogen (28 mM) as compared to MS medium (60 mM) and the molar ratio of NH_4^+/NO_3^- (1:10, as compared to 1:2 in MS medium). Nitrogen is essential to support cell growth and to feed the protein and nucleic acid synthesis, thus affecting secondary metabolism. In many cases, however, reducing the total nitrogen concentration in the medium leads to lower cell growth and higher product formation (Tigerstedt and Niskanen 2002). This could be an explanation for the high oil yield and gene expression observed in plants grown on SH. Also, this could justify the decrease observed in the carvacryl acetate, thymyl acetate and carvacrol methyl ether relative amounts in the essential oils of plants grown on SH medium. These compounds are formed as the result of post-enzymatic modifications of the primary structure of carvacrol and thymol, which are produced directly by terpene synthases (Keszei et al. 2008) and cytochrome P450 oxidases (Schuler and Werck-Reichhart 2003; Keeling and Bohlmann 2006). Carvacryl acetate and thymyl actetate were previously associated to stress response to *in vitro* conditions (Chapter II), likewise this could justify carvacrol methyl ether decrease in oils isolated from G1 and G2 genotypes grown on SH.

An increase in macronutrients, nitrogen and potassium, in *Salvia stenophylla* microplants enhanced accumulation of (-)-α-bisabolol (Musarurwa et al. 2012). The taxol production in cell cultures of *Taxus yunnanensis* was influenced by the nitrogen source and by its concentration in the medium (Chen et al. 2003). Similar results were observed in *Valeriana glechomifolia* plantlets, where valepotriate yieds also improved with the nitrogen concentration in the medium (Russowski et al. 2006). The reduction of total nitrogen in the culture medium resulted in approximately two fold increase of hypericins, in *Hypericum maculatum* (Coste et al. 2011). In roots suspension cultures of *H. perforatum*, the 5:25 (mM) NH_4^+/NO_3^- ratio was the optimum for biomass, total phenols and flavonoids accumulation (Cui et al. 2009). The reduced levels of NH_4^+ and increased levels of NO_3^- promoted the production of shikonin and betacyanins, whereas higher ratios of NH_4^+ to NO_3^- increased the production of berberine and ubiquinone (Dodds and Roberts 1985). For instance, in cell suspensions of *Panax ginseng*, saponin production was higher when an initial N concentration of 5–20 mM was used (Liu and Zhong 1997). In the same manner, suspension cultures of *Camptotheca acuminate* gave the maximum

camptothecin yield when a 5:1 NH_4^+/NO_3^- ratio was applied (Pan et al. 2004).

Phosphate concentration in the medium has a great effect on the production of secondary metabolites in plant cell cultures. Higher level of phosphate enhanced the cell growth, whereas it had a negative influence on secondary product accumulation (Smetanska 2008). Both culture medium, MS and SH, had a low concentration of PO_4^-, 1.25 mM in MS and 2.6 mM in SH. Medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product (Smetanska 2008). In *Beta vulgaris* hairy roots, it was found that phosphate was a key nutrient in pigment accumulation, and upon reduced phosphate levels (0-2.5 mM) higher pigment contents were obtained (Taya et al. 1994). Low phosphate content in the medium of *Hyosciamus muticus* root cultures limited growth, but increased sesquiterpene (solavetivone) production (Dunlop and Curtis 1991). In *Artemisia annua* hairy root cultures, phosphate was a quite powerful factor for both growth and production, 2.0 and 1.0 mM were optimal for root growth and artemisinic compounds, respectively (Weathers et al. 1997).

Potassium serves as a major contributor to osmotic potential, besides being needed for protein synthesis and for activation of particular enzyme systems (Zhong 2001). Recently, Green and collaborators concluded that potassium was necessary for α-farnesene synthase synthesis in apple. They proposed that potassium ions stabilize the H-α1 loop region for optimal substrate binding (Green et al. 2009). The increase of potassium in culture medium led to the accumulation of (-)-α-bisabolol in *S. stenophylla* (Musarurwa et al. 2012). SH medium has a higher amount of K^+ (25 mM) than MS medium (20 mM), however it was impossible to relate the expression of the terpene synthase genes with K^+ concentration. Furthermore, in the SC genotype the *Tctps2* and *Tctps4* gene expression was lower in plants grown on SH medium (Figs. 3 and 4).

5.2. Effect of fungal extract in shoot cultures

The response of the two genotypes, CT and SC, to *B. cinerea* macerate was very different and the exposure was toxic for the CT genotype, leading to plant death. *B. cinerea* is an airborne plant pathogen with a necrotrophic lifestyle (Williamson et al. 2007). Evidences suggest that pathogen attack could trigger the host to induce programmed cell death (PCD) as a defense strategy (Repka 2006; Williamson et al. 2007), which might be related to the observed necrosis in both *Thymus* genotypes despite using fungal extracts and not the living fungus. The stress level that a cell is exposed to can be determinant on the ultimate fate of the cell. In a population of cells subjected to low level stresses the majority will survive, when subjected to moderate levels of stress the majority execute PCD, while high levels of stress induce widespread necrosis (Reape et al. 2008). Elicitor concentration plays a very important role in elicitation process. The response to a particular elicitor

may vary from plant to plant and between different cells lines (Namdeo 2007). Namdeo and collaborators (2002) reported higher accumulation of ajmalicine in *Catharanthus roseus* cultures when treated with different concentrations of elicitor extracts of *Trichoderma viride*, *Aspergillus niger* and *Fusarium moniliforme*. Ajmalicine accumulation was higher in cells elicited with higher concentration (5%) of elicitor extracts as compared to lower concentration (0.5%). However, increasing the concentration further up to 10% adversely affected the accumulation of ajmalicine.

The growth/proliferation rate of CT and SC was affected by the presence of the fungal extracts, but in CT genotype the effect was more evident. The low growth rate could be a response to the reallocation of the energy resources. Tolerance and resistance traits require the reallocation of host resources from primary metabolism, since defensive chemicals are costly for plants and resistance genes might impose metabolic costs, resulting in a lower growth rate of the plants (Lattanzio et al. 2009). Almost all fungal elicitors stimulate the phenylpropanoid pathway, since this pathway leads to accumulation of phenolic compounds required for cell wall and salicylic acid biosynthesis and cell wall reinforcement is observed to be a basic defense strategy for plant cells against pathogen invasion (Zhao et al. 2005).

It was not easy to correlate terpene synthase gene expression with accumulation of a specific compound in the oils, since this species accumulates the essential oils in specialized structures, the glandular trichomes (Tholl 2006). It is usually quite difficult to study direct correlations between mRNA and levels of the respective terpenes because part of the essential oil content analyzed is already accumulated at a particular moment and does not reflect the rate of its synthesis at the time point analyzed (Schmiderer et al. 2010).

Fungal elicitation activates a range of plant defense related genes and finally leads to a series of defense reactions, including phytoalexin biosynthesis (Somssich and Hahlbrock 1998). In the biosynthetic pathway leading to terpenoid phytoalexins, terpene cyclases and prenyltransferases, have been shown to play a regulatory role (Chappell 1995; Hugueney et al. 1996; Bohlmann et al. 1998). Secondary metabolites with antifungal activity could be present constitutively in healthy plants, or they may be synthesized *de novo* in response to pathogen attack or other stress conditions (phytoalexins) (Dixon 2001; Ribera and Zuñiga 2012). In SC shoot cultures, an increase in carvacrol, thymol and their respective acetate compounds was observed in the oils of plants grown with fungal extracts. In CT shoots these were the main compounds found in the oils. Carvacol and thymol were shown to have strong activity against *B. cinerea* (Tsao and Zhou 2000; Bouchra et al. 2003; Camele et al. 2012). γ-Terpinene and sabinene in the gaseous state also showed high inhibitory activity against *B. cinerea* in *in vitro* assays (Espinosa-Garcia and Langenheim 1991). However, in this research study

the sabinene relative amount decreased when the shoots were transferred to MS medium with *B. cinerea* extracts.

In the literature there are several examples of induced terpene production and terpene gene expression following fungal stresses. *Cupressus lusitanica* cells induced by fungal stress emitted monoterpenes with a maximum on the second (sabinene and limonene) or third (terpinolene and β-ocimene) day, after which the concentration gradually declined. Elicited cells produced significant amount of β-thujaplicin from the 1st to 5th days after elicitation (Alwis et al. 2009). Treatment of *Papaver somniferum* cell suspensions with a homogenate of *Botrytis* mycelium resulted in a remarkable accumulation of sanguinarine of up to 3% (Constable 1990). Fungal endophytes showed to elicit taxol production in *Taxus* cell suspension cultures. Co-culturing of *T. chinensis* with *Fusarium mairei* fungus caused a 38-fold increase in a co-culture system (Li et al. 2009).

Elicitation of *Gossypium arboreum* suspension-cultured cells with a phytopathogenic fungus *Verticillium dahliae* induced transcription of both farnesyl diphosphate synthase and (+)-δ-cadinene synthase, paralleling the accumulation of the sesquiterpene aldehydes in these cells (Liu et al. 1999). The expression was higher in the first 12 hours after elicitation, but along the time course, the expression of both genes declined (Liu et al. 1999). The same was observed in *Citrus jambhiri* when infected with *Alternaria alternata*. The expression of *RlemTPS2* was slightly induced while the expression of *RlemTPS1* did not change. No expression was detected 24 hours after the treatment (Yamasake and Akimitsu 2007; Shishido et al. 2012). Down-regulation of D-limonene synthase by antisense techniques drovethe decrease of terpene content and it also up-regulated genes involved in the immune response against pathogens (*Penicillium digitatum*) in *Citrus sinensis* (Rodríguez et al. 2014). Recently, the volatiles and TPS transcripts accumulation were investigated in *Zea mays* upon infection with fungal pathogens of the Fusarium genus. In this study was observed emission of β-macrocarpene and β-bisabolene, which were correlated with an increased transcript accumulation of maize terpene synthase 6/11 (tps6/11) (Becker et al. 2014).

In the system described here, the expression of both γ-terpinene synthase genes was higher in the first 12 hours after transferring the shoots to the stress condition (MS+FE). The down-regulation of the terpene synthase genes observed one day after stress treatment could be explained by the transfer of the energy resources for more vital functions, such as induction of defense and resistance genes and primary metabolism.

6. Conclusion

In conclusion, *T. caespititius* shoot cultures proved to be a good experimental model to investigate the volatiles secondary metabolites. Controlled growth conditions reduce the sources of variability known to affect essential oil composition, thus making *in vitro* shoot cultures an advantageous system over the field natural conditions.

Culture medium composition showed to be important in terpene accumulation. Genotype susceptibility to *B. cinerea* extracts was higher in CT shoots compared to SC, however TPS gene expression was higher in SC, while in CT only a slight increase was observed. The influence of biotic and abiotic factors on the essential oils composition was revealed only on quantitative differences and seemed to be genotype dependent. The expression of the genes involved in the terpene synthesis was also influenced by the same factors, but a straight correlation between transcripts synthesis and essential oil profile could not be established.

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Chapter V

Final considerations

1. Conclusion

Thymus caespititius is an aromatic plant with distinctive chemotypes, namely thymol, carvacrol, α-terpineol, sabinene, and the mixed chemotypes thymol/sabinene, thymol/carvacrol and thymol/sabinene/carvacrol (Salgueiro 2006; Figueiredo et al. 2008; Trindade et al. 2008). These chemotypes are the result of natural evolution, since this species has never been exploited commercially and thus selective breeding has never been performed. The molecular mechanisms behind such chemical diversity are not yet clear.

In this study, the monoterpene biosynthesis in *T. caespititius* was investigated by molecular and biotechnological approaches. First, *in vitro* cultures from different chemotypes were established in order to have enough plant material, always available for the following studies (Chapter II). Then, the genes encoding enzymes of monoterpene biosynthesis were searched and identified in thyme and latter their expression level was assessed (chapter III). Finally, in chapter IV, some biotic and abiotic factors were assayed to study some aspects of the regulation of the monoterpene biosynthesis and accumulation in this aromatic species.

Together, these chapters provide a small contribution to the full understanding of how monoterpenes are biosynthesized and regulated in the Lamiaceae family.

In conclusion, the studies presented in this thesis showed that *in vitro* proliferated shoots resulting from axillary buds (C, CT and SC) produced essential oils qualitatively similar to the corresponding field-grown plants, apart minor quantitative differences. Considering the *in vitro* shoots obtained from seedlings (G1 and G2), a different essential oils profile was found in comparison with the mother-plant, due to the genetic variability.

The large biomass increase obtained *in vitro* opens the possibility of using large-scale production of desirable secondary metabolites or further adapting the system for obtaining plants with desired features (higher oil yield, new chemotype) that are capable of being transferred *ex vitro* and grown in the field.

Considering that *T. caespititius* essential oils are mainly composed by mono- and sesquiterpenes, research started by searching terpene synthase genes, allowing the identification and characterization of three *Tctps* genes in the five *in vitro* genotypes. Tctps2 and Tctps4 code both for a γ-terpinene synthase and due to the nucleotide sequence similarity they are most likely isogenes. Tctps5 in the presence of GPP produced α-terpineol as main product. The FPP feeding did not reveal any enzyme

activity. Although the enzymatic studies were only qualitative, it appears that temperature affects differently the three Tctps. At 4ºC no enzyme activity was detected for the recombinant Tctps2 from C genotype, whereas for the remaining constructs for Tctps2, enzyme activity remains unchanged for the temperatures tested (4ºC, 21ºC and 42ºC), the same occuring with Tctps4. Tctps5 protein activity seems to have a duality of product formation, which is temperature related.

Tctps4 was less expressed in thyme plants than *Tctps2.* γ-terpinene levels in aerial parts were reduced, probably due to the prompt conversion into carvacrol and thymol, the main components from the essential oils. *Tctps5* gene was only found in two genotypes, G1 and G2. Even in very low amounts, both genotypes had some α-terpineol in their essential oils.

The site-directed mutagenesis demonstrated that Arg-505 residue in Tctps2 is essential for protein stability allowing correct folding. The change for a nonpolar hydrophobic amino acid (Gly) resulted in a non-functional protein, probably because of disrupted stability.

It has been proven that biotic and abiotic factors can influence the essential oil composition as well as the genes involved in their biosynthesis. The work presented here also exploited how culture media composition and fungal extracts influence the terpene metabolism. Differential nutrient composition influenced quantitatively the essential oils content. A decrease in the acetate compounds in the essential oils was observed in shoots grown on SH medium when compared to shoots grown on MS. Assuming that acetate compounds are stress related (as suggested in Chapter II), this could indicate that on SH medium the plants are subjected to a less stressful condition. The terpene synthase transcripts accumulation was higher in all genotypes grown on SH, except in sabinene/carvacrol (SC) genotype. Nitrogen concentration and the NH_4^+/NO^{3-} ratio are factors that are quite different among both culture media, and one could hypothesize that they are important factors influencing the observed differences in the oils composition and in gene expression.

B. cinerea extract was applied on two thyme genotypes. This highly induced the expression of the *Tctps* genes in SC, but in CT only a slight increase was observed, showing clearly that the response was genotype dependent. Fungal extracts did not affect qualitatively and quantitatively the CT essential oils, however in SC, the sabinene relative content decreased and this was inversely related to the increase of two antifungal compounds, carvacrol and thymol, in the oil. Since *T. caespititius* accumulates the terpenes compounds at a specific time during plant development, it was very difficult to establish a correlation between transcripts synthesis with a specific compound accumulation.

The use of *in vitro* shoot cultures in studies on the production of essential oils is being explored in several Lamiaceae aromatic species. Controlled growth conditions reduce the sources of variability

known to affect essential oil composition, thus making *in vitro* shoot cultures an advantageous system. *T. caespititius* shoot cultures proved to be a good experimental model to investigate terpene metabolism, synthesis, regulation and accumulation and elucidate many of the questions.

2. Research Novelty

Thymus caespititius is an underutilized aromatic species, with some carvacrol- and thymol-rich chemotypes (Salgueiro 2006; Figueiredo et al. 2008; Trindade et al. 2008). These essential oils were used in several studies demonstrating their high antimicrobial (Dandlen et al. 2011a) and antioxidant properties (Miguel et al. 2003; Miguel et al. 2004; Dandlen et al. 2010), acetylcholinesterase inhibition (Dandlen et al. 2011b) and nematicidal (Barbosa et al. 2010; Barbosa et al. 2012; Faria et al. 2013) activities, principally those carvacrol- and thymol-rich. The phenolic monoterpenes, thymol and carvacrol, are two highly bioactive compounds found in thyme and other species. But despite the fact that thymol and carvacrol are widespread plant secondary compounds with important roles in plant defense, as well as flavors and pharmaceuticals, not much is known about their biosynthesis.

This work has provided a molecular and biotechnological resource for this non-model species which will aid future efforts on terpene synthases studies and to improve some thyme features (oil yield, new chemotypes) with potential applicability in the industries. The *in vitro* cultures established here opens up the possibility of using large-scale *in vitro* production, without environmental constrains. This may be of particular interest for the study of carvacrol and thymol biosynthesis, since both compounds are present in high amount in some essential oils. Also, with the increasing interest in aromatic plant by the consumers, as spice and as herbs for salt substitute, this species could be exploited commercially.

The TPS genes study in *T. caespititius* is very recent and in this work it is reported for the first time a second gene encoding for γ-terpinene synthase. The lack of transit peptide in *Tctps4* indicates that the subcellular localization of this gene might be cytosolic, while the *Tctps2* is plastidial.

It is known that enzyme activity depends of the optimal temperature for its proper functionality. For TPS several temperatures, varying from 15ºC and 42ºC, have been tested and used in enzymatic assays (Deguerry et al. 2006; Crocoll et al. 2010; Demissie et al. 2011; Krauser et al. 2013; Lima et al. 2013; Lin et al. 2014). However, in the present study for the first time enzymatic assays were performed at 4ºC, revealing monoterpene synthase activity at this temperature.

Also, it is the first time that two TPS genes regulatory factors, nutrients and fungus extracts are tested on *Thymus* species. Likewise, it was not found any report on the biotic or abiotic influences on terpene metabolism on any species of this genus.

3. Assumptions and Limitations

Although it is relatively easy to establish *in vitro* cultures from seeds, the same does not happen when axillary buds are used as starting material. For this study the *in vitro* establishment from axillary buds was attempted, but this was not possible for all the selected chemotypes. The α-terpineol type was impossible to establish, due to the high contamination rate observed (92-100%). So, in an attempt to have this chemotype *in vitro,* seeds from an α-terpineol plant were used as starting material. However, the use of seeds does not ensure the establishment of a specific plant chemotype, due to genetic variability. Although the genetics of chemotype inheritance is mostly unknown, one would expect that seeds derived from an α-terpineol chemotype might have a high amount of α-terpineol in the essential oils. Unfortunately, this was not observed and although this monoterpene level was higher than in the other chemotypes, it was not similar to the abundance found in the mother-plant.

Eight *T. caespititius in vitro* cultures were established, but only five were selected after analysis of the chemotype stability and plant fitness to proceed with this study.

Another difficulty found in this work is related to the sabinene synthase. Several attempts were made to find this gene, including alignments between sabinene synthases of *O. vulgare* and *Salvia* spp., but the fragments obtained always aligned with *Tctps2* and *Tctps4*. This information suggests that the sabinene synthase could be very similar to the three genes already identified in *T. caespititius* and the approaches used here were didn't allowed its identification. As referred by several authors, the knowledge of the amino acid sequence of a terpene synthase is not sufficient to predict which terpene(s) will be produced (Fisher et al. 2013). The main reason for terpene diversity is that terpene synthases are evolutionarily plastic enzymes that readily change and thereby acquire new catalytic properties (Trapp and Croteau 2001). A wide variety of residue changes can alter how active site residues interact with the substrate or reaction intermediate(s), thereby impacting product structure (Segura et al. 2003; Greenhagen et al. 2006; Keelling et al. 2008).

In the work presented here, no kinetic studies on the monoterpene synthases identified were performed. All the enzyme activity data presented here are qualitatively data. So, the proper enzyme activity was not determined, as well the factors that could affect it such as co-factor type, pH and temperature.

4. Future Directions

Despite the increasing demands and tremendous importance of plant secondary products for human consumption (drugs, flavors and fragrances), as well as decades of research on the area, much of plant secondary metabolism is still poorly understood.

Some of the key questions to address include:

4.1. How is chemotype inherited?

Few studies were made in this area, one in *T. vulgaris*. Despite being a species from the same genus as *T. caespititius* some of the characterized chemotypes are different. So, no straight correlation can be transposed from *T. vulgaris* to *T. caespititius* and it is necessary to understand how chemotype is inherited in *T. caespititius*, as well as in other plant species with chemical polymorphism. Inheritance studies should be made, involving crosses between plants with different chemotypes and progeny' essential oil evaluation to determine the genetic control of this feature.

4.2. Could *T. caespititius in vitro* cultures be a source of secondary metabolites?

Plants *in vitro* systems are considered eco-friendly for the production of biomass and secondary metabolites and have great commercial potential (Marchev et al. 2014). The manipulation of *in vitro* cultures to improve productivity of target compounds can be made, through improving chemical processing and bioreactor performance or employing elicitors, abiotic stresses, and other approaches.

Large-scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemicals independent of plant availability (Debnath et al. 2006).

As observed in Chapters II and IV, *T. caespititius in vitro* cultures produced more oil yield than field-grown plants, and some of those oils are carvacrol- and thymol-rich, two components with biological activities. The manipulation of a few parameters in thyme shoot cultures could enhance the production of desire compounds, as well the synthesis of new compounds.

In this thyme species, terpenes were the main group of secondary metabolites studied, so probably this species could be rich in other interesting components. If so, these cultures could be used for the synthesis of those compounds.

4.3. *T. caespititius* essential oils could be used in the control of pests and diseases?

Pest management products based on plant material, plant extracts, or natural products derived from plants, have long been touted as potential alternatives to conventional synthetic pesticides, presumably because the natural products would have lesser environmental and human health impacts than many of the older conventional pesticides that had demonstrable adverse effects on non-target organisms and ecosystems (Isman et al. 2011).

There are already available on the market several pesticides based on oils from clove leaf (*Syzygium aromaticum*, Myrtaceae), rosemary (*Rosmarinus officinalis*, Lamiaceae), peppermint (*Mentha* x *piperita*, Lamiaceae), cinnamon leaf (*Cinnamomum zeylandicum*, Lauraceae), lemongrass (*Cymbopogon nardus*, Poaceae) and thyme (*Thymus vulgaris*, Lamiaceae) (Isman et al. 2011).

Essential oils from some of *Thymus caespititius* chemotypes could be used in the biopesticides formulations like the other aromatic plants. Thymol and carvacrol-rich essential oils proved to have high antimicrobial activity (Dandlen et al. 2011a). More recently, *T. caespititius* essential oils were screened for their nematicidal activity against the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Barbosa et al. 2010; Barbosa et al. 2012; Faria et al. 2013). Also the two phenol compounds, carvacol and thymol already showed a strong antifungal activity on *B. cinerea* (phytopathogenic fungi) (Camele et al. 2012).

4.4. Which other genes may be involved in terpene biosynthesis in *T. caespititius*?

T. caespititius essential oils are mainly composed by mono- and sesquiterpenes. So far, only three genes were identified and many more remain to be identified, like sabinene synthase gene and sesquiterpene synthase genes.

TPS are often suggested to have molecular promiscuity resulting from plasticity residues that slightly change the template or carbocation stabilization when the amino acid is changed and thus direct product outcome (Fisher et al. 2013). Considering the efforts to find the sabinene synthase in this species, which always resulted on γ-terpinene amplification, the several TPS genes present in *T. caespititius* must be very similar rendering their search difficult. So, site-directed mutagenesis and domain swapping are good methods to target and identified specific residues responsible for product specificity (Hyatt and Croteau 2005; Fischer et al. 2013).

Transcription factors (TF) and cytochrome P450 are two important gene families involved in terpene metabolism with great interest and they should be studied in *T. caespititius*.

Relatively few transcription factors that regulate terpenoid biosynthesis have been identified to date (De Geyter et al. 2012). So far TFs have been identified in *Arabidopsis* (Hong et al. 2012), *Artemisia*

annua (Ma et al. 2009; Yu et al. 2012; Lu et al. 2013), *Catharanthus roseus* (Suttipanta et al. 2011; Zhang et al. 2011), *Gossypium arboretum* (Xu et al. 2004); *Nicotiana attenuata* (Skibbe et al. 2008) and more recently in *Solanum lycopersicum* (Spyropoulou et al. 2014).

Cytochromes P450 catalyzes the decoration (hydroxylation, dehydrogenation, isomerization, and several more) of the basic terpene skeletons and thereby contribute significantly to the structural diversity observed in the terpene group (Weitzel and Simonsen 2014). Discovery of pathway specific cytochromes P450 and their involvement in terpenoid biosynthesis require thorough biochemical characterization (Weitzel and Simonsen 2014). Monoterpenoid biosynthesis and cytochromes P450 involved therein have been studied in-depth in plants belonging to the Lamiaceae family. Both peppermint (*Mentha* x *piperita*), spearmint (*M. spicata*), and perilla (*Perilla frutescens*) express limonene hydroxylases, (Lupien et al. 1999; Mau et al. 2010; Weitzel and Simonsen 2014). Recently, five new members of the CYP71D subfamily have been cloned from oregano (*O. vulgare*) and thyme (*T. vulgaris*) (Crocoll 2011; Weitzel and Simonsen 2014). All these information could be useful to conduct the search of these enzymes in *T. caespititius*.

In recent years, genomic approaches have been widely used for discovering and characterizing secondary metabolism pathways and the related genes in several plant species (Yang et al. 2013, Galata et al. 2014). Next-generation sequencing (NGS) platforms provide highly efficient tools to discover novel enzymes and transcription factors from non-model species. Profiling *T. caespititius* transcriptome will help the identification of new genes involved in terpene biosynthesis. However, this is an expensive technique, and a preliminary approach would be to use the information from related species (like salvia) whose transcriptome has been already analyzed using this platform.

4.5. Which biotic or abiotic factors could regulate the TPS expression in *T. caespititius*?

Profiling all or a group of secondary metabolites from plants under various treatments will help to understand metabolic fluxes and the related regulatory mechanisms. Transcript analysis of plants under different stresses, such as bacteria, MeJA, JA, UV-B radiation, water stress and several others, could display differential regulation of plant secondary metabolism-related genes in an individual plant. This was already started using different *T.caespititius* chemotypes as reported here, but other stresses as mentioned above could be also tested in thyme to fully address the question of terpene synthesis in these plants.

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